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14. ABSTRACT Breast cancer is the second leading cause of cancer death in Canadian women. To metastasize, cells must move through the stroma of the breast, enter the circulation, survive transit, exit the circulation, and form a secondary tumor. It is not fully understood how breast cancer cells gain the ability to move or what signaling pathways mediate these events, and identification of critical components of these pathways would represent potential targets for anti-metastatic therapies. The MUC1 glycoprotein is expressed on the apical membrane of normal breast epithelia. In many human breast carcinomas, MUC1 is overexpressed and loses apical polarization, events that correlate with increased metastasis. Several critical steps of the metastatic cascade require cell adhesion, and it has been reported that MUC1 is a ligand for ICAM-1, which is expressed throughout the migratory tract of a metastasizing breast cancer cell. It was subsequently reported that MUC1/ICAM-1 binding initiates calcium oscillations, cytoskeletal reorganization, and cell migration, suggesting that binding could be important in metastasis. Here, we investigate the mechanism of MUC1/ICAM-1 binding induced signaling. We show that MUC1 forms constitutive dimers which are required for Src recruitment and ICAM-1 binding induced signaling. We show that MUC1 dimers are not covalently linked and do not require cytoplasmic domain cysteine residues. These results reveal information on the mechanism of MUC1/ICAM-1 signalling, which can be used to identify novel targets and combinational strategies for anti-metastatic therapy in breast cancer.					
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Introduction

Breast cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related mortality in Canadian women with an estimated 5,300 deaths in 2010 [1]. In these patients, mortality was due to metastasis of cells from the primary breast tumor to distant sites such as bone, liver, and brain, where metastatic tumors form, leading to impairment of organ function [2, 3]. In order to generate secondary tumors, cancer cells must complete several steps in the metastatic cascade, including movement through the stroma, entry into circulation, survival during transit, and movement through endothelial cells and the basement membrane at distant sites. A key requirement during this process is cell motility, a tightly orchestrated process involving numerous cell signalling pathways, actin cytoskeletal

reorganization, and disruption and formation of focal adhesions between the migrating cell and underlying substratum [4]. It is not clear how breast cancer cells develop the ability to move, or what proteins and signalling pathways mediate each step. Clarification of the mechanism(s) underlying the inappropriate or persistent activation of migratory signalling in breast cancer cells is a critical step in the development of therapies which can be used clinically to reduce breast cancer related mortality.

The MUC1 transmembrane glycoprotein is expressed on the apical membrane of normal breast epithelia and consists of a heavily glycosylated extracellular domain (ECD), single pass transmembrane domain (TMD), and a 72-amino acid (aa) cytoplasmic domain (CD). In many human breast carcinomas, MUC1 is overexpressed, underglycosylated and loses apical polarization, events that correlate with increased metastasis [5-7]. A large body of research has emerged investigating the role of MUC1 in carcinogenesis, cell survival, motility, and growth, with several promising clinical developments investigating the potential for MUC1 in cancer detection and therapy [8-10]. However, the correlation between increased MUC1 expression and increased metastasis is not completely explained by the majority of existing studies that attribute an anti-adhesive role to MUC1 due to its large, negatively charged ECD. Although anti-adhesive properties would contribute to cell motility at certain points in cancer metastasis, other steps require cell-cell adhesion, a role that is at odds with MUC1's proposed anti-adhesive function. In this regard, we were the first to report that MUC1-ECD binds to ICAM-1, a protein present on activated stromal and endothelial cells, key points in the path of a metastasizing breast cancer cell [11, 12]. Subsequently, we reported that MUC1/ICAM-1 binding leads to generation of cellular calcium oscillations (CaOs), cytoskeletal rearrangements, and cell motility in MUC1-transfected cells and in MUC1 expressing breast cancer cells [13-15]. The mechanism of MUC1/ICAM-1 induced signalling has not been fully described, although our previous studies implicated the activity of Src kinase, a non-receptor tyrosine kinase, in transduction of the ICAM-1 signal. Although the mechanism of Src recruitment and activation is unclear, Src targeted anti-cancer therapies have been developed and tested clinically, and there is great potential for the rational combination of drugs targeting multiple components of a single pathway [16-18]. Therefore, investigation of the role of Src kinase in transmission of the

MUC1/ICAM-1 signal may lead to development of therapeutic strategies which effectively target breast cancer metastasis.

In recent years, a body of research has emerged indicating that MUC1 forms dimers which are dependent on membrane proximal cysteine residues [19]. Inhibitors of MUC1 dimerization have been shown to result in cancer cell death *in vitro* and *in vivo* [20-22], although the mechanism of MUC1 dimerization and the role of dimerization in other cell processes, such as metastasis, is unclear. As our reported MUC1 ligand, ICAM-1, exists as a dimer, it is plausible that MUC1 dimerization plays a role in transmission of the ICAM-1 binding induced signal. Investigation of the role of MUC1 dimerization in transmission of the ICAM-1 signal could reveal additional clinical applications for the inhibitors of MUC1 dimerization already in existence, as well as provide targets for the development of novel therapies.

In this study, we investigate the importance of Src kinase and MUC1 dimerization in transmission of the ICAM-1 binding induced signal. We report that Src kinase is a critical component of the MUC1/ICAM-1 signalling pathway, highlighting the importance of investigation of the mechanism of Src recruitment and activation. MUC1 dimers form constitutively, are not covalently linked, and are independent of membrane proximal cysteine residues, contrary to previous reports. Inhibition of MUC1 dimerization results in decreased Src binding and disruption of ICAM-1 binding induced CaOs and cell migration. Importantly, we report that MUC1 dimerization is required for Src recruitment and ICAM-1 binding induced events, a novel and clinically significant finding.

Body

Src is required for MUC1/ICAM-1 binding induced events

Following treatment with Lipofectamine only, Scramble siRNA, or Src siRNA, parental and MUC1-CFP transfectants were assayed for the previously described ICAM-1 binding induced CaOs [13] (Fig 3.4). As parental cells do not express MUC1, the 293T Lipofectamine only condition was set to one as a negative control. All conditions using parental cells resulted in equivalent levels of CaOs, indicating

that Src knockdown does not affect the magnitude of CaOs generated in response to ICAM-1 treatment in these cells. In MUC1-transfected cells which were treated with Lipofectamine only, a statistically significant increase in Oscillation Factor after ICAM-1 stimulation was observed compared to parental conditions, in agreement with prior publications describing the MUC1/ICAM-1 binding induced CaOs [13]. Treatment with Scramble siRNA did not result in a significant change in the oscillation factor for MUC1-CFP cells, but treatment with Src siRNA decreased the oscillation factor significantly compared to Lipofectamine only or Scramble siRNA treatments, was still significantly greater than negative control conditions.

Cells were also assayed for transmigration through an ICAM-1 positive monolayer, a previously described phenomenon following MUC1/ICAM-1 binding [15] (Fig 3.5). Again, parental Lipofectamine only treatment condition was set to one and the remaining conditions expressed as a ratio. Parental cell migration was not affected by siRNA treatment, indicating that MUC1 expression is required to elicit ICAM-1 binding mediated CaOs. In MUC1-transfected cells treated with Lipofectamine only, a significantly greater number of cells migrated compared to this control, in agreement with prior data demonstrating that MUC1/ICAM-1 binding results in cell motility and migration [14, 15]. In MUC1-CFP cells, Scramble siRNA did not significantly affect migration levels compared to Lipofectamine only. Treatment of MUC1-CFP cells with Src siRNA significantly reduced the level of cell migration compared to MUC1-CFP Lipofectamine only or Scramble siRNA, but there was still a significantly greater number of cells migrated than parental negative control conditions.

MUC1 forms constitutive cytoplasmic domain dimers in human breast cancer cell lines and transfected HEK 293T cells.

MUC1 positive human breast cancer cell lines MCF-7 and T47D (Fig 4.1A) and HEK 293T cells transfected with MUC1-CFP (Fig 4.1B) or the MUC1 splice variant lacking the tandem repeat domain MUCY-YFP-Fv (Fig 4.1C) were lysed with or without prior treatment with the membrane permeable crosslinker DSS. SDS-PAGE and probing with anti-MUC1-CD revealed

the invariable appearance of a new MUC1-CD species at exactly double the molecular weight of the monomeric cytoplasmic domain after treatment with DSS, consistent with the presence of a MUC1-CD homodimer. The appearance of MUC1-CD dimers in MUCY-YFP-Fv transfectants indicates that the tandem repeat domain is not required for dimerization.

We then investigated the contribution of the MUC1 cytoplasmic domain to dimer formation. HEK 293T cells were transfected with MUCY-YFP-Fv and/or CD8/MUC1, described in [23], a chimera of CD8 extracellular and transmembrane domains and MUC1-CD domain, beginning at R⁴RK (lacking C¹QC motif). Single transfection of either CD8/MUC1 or MUCY-YFP-Fv and probing with anti-MUC1-CD revealed their molecular weights to be approximately 40 and 75 kDa, respectively (Fig 4.2, lanes 1 and 2). Immunoprecipitation of the CD8/MUC1 + MUCY-YFP-Fv double transfectant (Fig 4.2, lane 3) with anti-CD8 and probing with anti-MUC1-CD (Fig 4.2, lane 4) resulted in the appearance of a 75kDa MUC1-CD species on a Western blot (Fig 4.2, red square), consistent with the molecular weight of MUCY-YFP-Fv. This indicates an association between CD8/MUC1 and MUCY-YFP-Fv. This association is significant because the CD8/MUC1 construct only contains the cytoplasmic portion of MUC1, beginning at R⁴RK, and does not contain the C¹QC motif, fluorescent tags, or the Fv domain. Therefore, association between these two entities must be due to the MUC1 cytoplasmic domain. Taken together, these data show that MUC1-CD forms constitutive cytoplasmic domain dimers which are not dependent on the VNTR domain, TMD, the C¹QC motif, or engineered C-terminal tags.

MUC1 cytoplasmic domain dimerization can be disrupted by addition of an engineered Fv domain and a monomeric Fv domain ligand.

To investigate the importance of MUC1 dimerization in Src association and ICAM-1 induced signalling, we sought to manipulate dimerization using a construct of MUC1 containing a C-terminal Fv domain (ARIAD Pharmaceuticals), and bivalent (AP20187^D) or monovalent (AP21998^M) ligands. Dimerization of Fv domain containing proteins can be manipulated by addition of Fv domain ligands. Previously, this system has been used to successfully manipulate dimerization of growth factor receptors [24] and G protein-coupled receptors [25]. Mechanistically, the bivalent ligand, which contains two Fv-binding

domains, effectively brings two Fv-domain containing proteins within close proximity – “dimerization”. The monovalent ligand, which contains one Fv-domain binding domain, is designed to bind to Fv-domain containing proteins and sterically inhibit their interaction with other Fv-domain containing proteins – “disaggregation or “monomerization” (Fig 4.3A). We found that treatment of 293T MUC1-CFP-FvHA cells for one minute with increasing concentrations of AP20187^D did not increase the quantity of MUC1-CD dimers above baseline levels, while AP21998^M treatment resulted in a dose dependant reduction in the level MUC1-CD dimers (Fig 4.3B). Densitometric analysis of the dimer bands normalized to total MUC1-CD illustrates the change in proportion of MUC1 in dimer form with AP21998^M treatment (Fig 4.3C). As a control, 293T MUC1-CFP cells, which lack the Fv domain, do not show a significant change in dimer quantity following treatment with 1 uM AP20187^D or AP21998^M (Fig 4.3D).

Disruption of MUC1-CD dimerization does not result in loss of cell viability.

As previous reports [26] have demonstrated that disruption of MUC1-CD dimerization using peptides results in arrest of cell growth and necrotic cell death, we performed a trypan blue exclusion viability assay after treatment with 1uM AP20187^D or AP21998^M and saw no significant reduction in viability, compared to no treatment control, up to 72 hours exposure (Fig 4.4).

Disruption of MUC1-CD dimerization results in decreased recruitment of total and active Src kinase to MUC1-CD.

To determine the importance of MUC1-CD dimerization in constitutive Src recruitment, 293T MUC1-CFP-Fv (Fig 4.5A), and, as a control, 293T MUC1-CFP (Fig 4.5 B) cells were treated with increasing concentrations of AP20187^D or AP21998^M for one minute, followed by immunoprecipitation with anti-MUC1-CD. Following separation on SDS-PAGE, blots were probed with anti-Src (total Src) and anti-Src^{P-Y416} (active Src). In the MUC1-CFP-Fv transfectants, the amount of total and active Src associated with MUC1-CD decreased in a dose-dependent manner with AP21998^M treatment (Fig 4.5A,

arrows). Treatment with AP20187^D did not result in a significant change, and Src recruitment to MUC1-CFP (Fig 4.5B) was not affected by Fv ligand treatment.

Densitometric analysis of Src and Src^{P416} normalized to MUC1-CD illustrates these results (Fig 4.6).

Disruption of MUC1-CD dimerization results in decreased ICAM-1 binding induced calcium oscillations and cell migration.

To determine if MUC1-CD dimerization is important in the previously observed ICAM-1 binding induced events, we assayed for ICAM-1 binding induced CaOs and invasion through an ICAM-1 positive monolayer after addition of the Fv ligands 1uM AP20187^D or 1uM AP21998^M and compared this to a no treatment control. 293T MUC1-CFP-Fv and, as controls, the Fv-domain negative 293T MUC1-CFP cells and the MUC1-negative 293T (parental) cells, were assayed for ICAM-1 binding induced CaOs (Fig 4.7) and invasion through an ICAM-1 monolayer (Fig 4.8). For each experiment, the parental no treatment condition was set to one and the remaining experiments expressed as ratios. MUC1-CFP and MUC1-CFP-Fv transfected cells displayed significant and statistically equivalent increases in CaOs and migration compared to control, in no treatment conditions. This indicates that the presence of the CFP and Fv domains do not interfere with the generation of ICAM-1 binding induced signalling. We found that ICAM-1 binding induced CaOs and invasion in 293T MUC1-CFP-Fv cells was significantly reduced after treatment with AP21998^M, compared to no treatment control. However, CaOs levels were still significantly greater than those observed in Parental conditions. Treatment with AP20187^D resulted in a significant increase in cell migration in 293T MUC1-CFP-Fv cells (Fig 4.8), but did not produce a significant response in the CaOs assay (Fig 4.7). Addition of the Fv domain ligands had no significant effect on the 293T MUC1-CFP transfectants lacking the Fv domain or parental cells lacking MUC1 expression.

MUC1-CD dimers are not covalently linked

As prior reports [19] have demonstrated that truncated MUC1-CD dimers are covalently linked *in vitro*, we investigated the formation of covalently-linked MUC1-CD dimers *in vivo* before and after

ICAM-1 stimulation. By omitting the reducing agent β -mercaptoethanol from the LSB used to prepare lysates for SDS-PAGE, we expected that covalently linked species would remain intact. Reducing (R, + β -mercaptoethanol) and non-reducing (NR, no β -mercaptoethanol) samples were run on separate SDS-PAGE gels as leaching of β -mercaptoethanol can occur. In both human breast cancer MCF-7 cells and 293T MUC1-CFP cells, no evidence of covalently linked MUC1-CD dimerization was observed constitutively (Fig 4.9A) and following stimulation with NIH ICAM-1 cells for 60 seconds (Fig 4.9B) when probed with anti-MUC1-CD. As a control for our technique, 293T CD8/MUC1 transfectants, which are expected to exist as covalently linked dimers via a CD8-ECD bridge [27, 28], were run under reducing and non-reducing conditions, revealing the presence of a covalently linked species at the molecular weight expected for a CD8/MUC1 dimer (Fig 4.9C).

MUC1-CD contains SH2 and SH3 binding domains which act to recruit Src kinase

To further reveal the mechanism of Src binding to MUC1-CD, we mutated the confirmed Src SH2 binding site (Y⁴⁶F; Δ SH2) [29] and/or the putative Src SH3 binding site (P³⁷A/P³⁸A; Δ SH3). As discussed in Section 1.2.5., Src SH3 domain binding is ideally suited to the motif “PXXP”, with arginine residues lying either N- or C-terminal to the polyproline motif [30], although there are many examples of Src SH3 domain binding motifs lacking this sequence [31]. As Src SH3 domain has been shown to bind MUC1-CD as an undescribed motif [29], the MUC1-CD sequence “R³⁴YVPPSSTDR⁴³” is the most likely SH3 binding site, containing both proline and arginine residues. We mutated the SH2 and/or putative SH3 binding domains on the MUC1-CFP-Fv plasmid, transfected HEK 293T cells, and following immunoprecipitation with anti-MUC1-CD, probed for Src and, as a loading control, MUC1-CD (Fig 4.10). We found that mutation of either the SH2 or the SH3 binding domain in MUC1-CD resulted in a decrease in the level of Src recruited to MUC1-CD, although MUC1-CD and Src were still associated. When both the SH2 and SH3 domain were mutated, Src recruitment to MUC1-CD was not detectable. These data indicate the MUC1-CD may recruit Src constitutively by both the SH2 and SH3 binding domains, and when one is mutated recruitment by the other is not affected. When both binding domains

are mutated, Src is not recruited to MUC1, indicating that the SH2 and SH3 binding domains are the only Src recruitment motifs present on MUC1-CD.

Src recruitment is not required for MUC1-CD dimerization

To determine if MUC1-CD dimerization is dependent on recruitment of Src kinase, we assayed dimerization in MUC1-CFP-Fv cells with SH2 and/or SH3 domains mutated (Fig 4.11). As described below, MUC1-CFP-Fv Δ SH2 and MUC1-CFP-Fv Δ SH3 display reduced recruitment of Src compared to wildtype, while MUC1-CFP-Fv Δ SH2/SH3 does not recruit Src. Following treatment with DSS, cells were run on SDS-PAGE and probed with anti-MUC1-CD. MUC1-CFP-Fv Δ SH2, MUC1-CFP-Fv Δ SH3 and MUC1-CFP-Fv Δ SH2/3 all formed MUC1-CD dimers (Fig 4.11), indicating that Src recruitment to MUC1-CD is not necessary for dimerization.

To further investigate the requirement for Src kinase in MUC1-CD dimerization, we utilized mouse embryonic fibroblasts (MEF) with *Src/Yes/Fyn* triple gene knockout (SYF^{-/-}). Transfection of MUC1-CFP-Fv and treatment with DSS revealed that MUC1-CD forms dimers in MEF SYF^{-/-} cells lacking SFKs (Fig 4.12). This confirms the results shown in Fig 4.11, demonstrating that MUC1-CD dimerization is not dependent on Src kinase recruitment. Transfection of Src or Y530F Src, a constitutively active mutant, did not significantly affect MUC1-CD dimerization in MEF SYF^{-/-} cells, indicating that the presence of Src does not potentiate MUC1-CD dimerization. Taken together, these data show that MUC1-CD dimerization occurs independently of Src, as Src is not required to be bound to MUC1-CD or present in the cell for dimerization to occur.

Key Research Accomplishments

- Demonstrated that Src is required for MUC1/ICAM-1 binding induced events, indicating a potential for synergistic therapies
- Demonstrated that MUC1-CD forms constitutive dimers

- Developed a system to disrupt MUC1-CD dimerization through addition of an engineered Fv domain and a monomeric Fv domain ligand.
- Demonstrated that MUC1-CD dimerization is required for Src recruitment and ICAM-1 binding induced cell motility

Reportable Outcomes

- This study demonstrated that MUC1, a protein overexpressed in breast cancer and implicated in breast cancer metastasis, functions partially through the formation of cytoplasmic domain dimers. These dimers are required for MUC1 to interact with key downstream signaling partners, and are critical for the initiation of cell motility following binding to ICAM-1. These results identify potential targets for anti-metastatic therapies in breast cancer patients.
- Results of this study were included in the following manuscript, which was accepted for publication in August 2011:

Ashlyn J Bernier, Jing Zhang, Erik Lillehoj, Andrew R.E. Shaw, Nirosha Gunasekara , Judith C Hugh: **Non-cysteine linked MUC1 cytoplasmic dimers are required for Src recruitment and ICAM-1 binding induced cell invasion.** *Molecular Cancer* 2011, **10**:93.
- The principal investigator, Ashlyn Bernier, completed the requirements for her PhD while receiving funding from this grant.

Conclusions

The data presented in this chapter demonstrate the role of MUC1-CD dimerization in ICAM-1 binding induced signalling, a proposed step in breast cancer metastasis, as well as reveal information on the mechanism of MUC1-CD dimerization. MUC1-CD dimerization occurs in both human breast cancer cell lines and transfected HEK 293T cells, indicating that dimerization is not dependent on a malignant phenotype. Further, we demonstrate that MUC1-CD dimerization occurs independent of the tandem repeat domain, and the association of MUC1-Y and CD8/MUC1, which contains only 69aa of the MUC1-

CD, indicates that dimerization occurs due to cytoplasmic interactions independent of the C¹QC motif. We also demonstrate that MUC1-CD dimerization in cells is not due to covalent bonding, contrary to other reports [19]. The role of the C¹QC motif in MUC1-CD dimerization and signalling will be investigated further in Chapter 5. Our disruption of MUC1-CD dimerization using the engineered “Fv domain” and monovalent Fv ligands allowed for investigation of the role of dimerization in recruitment of Src kinase and our previously described ICAM-1 binding induced CaOs and cell migration. As we have shown in Chapter 3 that Src kinase is a critical component of the MUC1/ICAM-1 signalling axis, we investigated the effect of MUC1-CD dimerization on recruitment of Src. We found that disruption of dimerization prevented both Src recruitment and ICAM-1 binding induced events. This finding is novel and significant, as inhibition of ICAM-1 binding induced cell migration represents a potential target in anti-metastatic therapies for breast cancer. Also, our findings showing that disruption of Src binding and ICAM-1 binding induced signalling are both inhibited by interference with MUC1-CD dimerization suggest that Src kinase is a direct modulator of the MUC1/ICAM-1 signal, not an indirect, downstream component. Lastly, we demonstrate that MUC1-CD dimerization occurs independently of Src binding, as MUC1-CD existed as a constitutive dimer in the absence of Src binding, through mutation of Src SH2/SH3 binding domains or knockout of Src. The data presented in this chapter shed light on the mechanism of Src recruitment to MUC1 and ICAM-1 binding induced signalling, representing potential targets for anti-metastatic therapies in the future.

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RESEARCH

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Non-cysteine linked MUC1 cytoplasmic dimers are required for Src recruitment and ICAM-1 binding induced cell invasion

Ashlyn J Bernier¹, Jing Zhang¹, Erik Lillehoj², Andrew RE Shaw³, Nirosha Gunasekara¹ and Judith C Hugh^{1*}

Abstract

Background: The mucin MUC1, a type I transmembrane glycoprotein, is overexpressed in breast cancer and has been correlated with increased metastasis. We were the first to report binding between MUC1 and Intercellular adhesion molecule-1 (ICAM-1), which is expressed on stromal and endothelial cells throughout the migratory tract of a metastasizing breast cancer cell. Subsequently, we found that MUC1/ICAM-1 binding results in pro-migratory calcium oscillations, cytoskeletal reorganization, and simulated transendothelial migration. These events were found to involve Src kinase, a non-receptor tyrosine kinase also implicated in breast cancer initiation and progression. Here, we further investigated the mechanism of MUC1/ICAM-1 signalling, focusing on the role of MUC1 dimerization in Src recruitment and pro-metastatic signalling.

Methods: To assay MUC1 dimerization, we used a chemical crosslinker which allowed for the detection of dimers on SDS-PAGE. We then generated MUC1 constructs containing an engineered domain which allowed for manipulation of dimerization status through the addition of ligands to the engineered domain. Following manipulation of dimerization, we immunoprecipitated MUC1 to investigate recruitment of Src, or assayed for our previously observed ICAM-1 binding induced events. To investigate the nature of MUC1 dimers, we used both non-reducing SDS-PAGE and generated a mutant construct lacking cysteine residues.

Results: We first demonstrate that the previously observed MUC1/ICAM-1 signalling events are dependent on the activity of Src kinase. We then report that MUC1 forms constitutive cytoplasmic domain dimers which are necessary for Src recruitment, ICAM-1 induced calcium oscillations and simulated transendothelial migration. The dimers are not covalently linked constitutively or following ICAM-1 binding. In contrast to previously published reports, we found that membrane proximal cysteine residues were not involved in dimerization or ICAM-1 induced signalling.

Conclusions: Our data implicates non-cysteine linked MUC1 dimerization in cell signalling pathways required for cancer cell migration.

Background

The ability of malignant cells to escape from a primary tumour mass and migrate to distal sites to form metastatic tumors is the cause of mortality in the majority of carcinomas, including breast carcinoma. Approximately 20% of breast cancers belong to the Luminal B genetic subtype, typified by estrogen receptor positivity and a slow, steady rate of recurrence over time despite anti-

estrogen therapy [1]. Estrogen is known to increase the expression of MUC1 [2], a well-characterized member of the mucin family of glycoproteins, and a correlation has been demonstrated between MUC1 expression, resistance to anti-estrogen therapy and metastatic behaviour [3]. We have been investigating the mechanism of cell migration in the Luminal B breast cancer cell lines MCF7 and T47D, and were the first to demonstrate that MUC1 mediates heterotypic cell-cell adhesion by binding ICAM-1 [4], which is expressed on peritumoral stromal and endothelial cells. Subsequently, we demonstrated that ICAM-1 binding triggers calcium

* Correspondence: judith.hugh@albertahealthservices.ab.ca

¹Department of Laboratory Medicine and Pathology, 3-70 Heritage Medical Research Centre, University of Alberta, Edmonton, AB, T6G 2S2, Canada
Full list of author information is available at the end of the article

oscillations which may activate proteins involved in focal adhesion disassembly and cell contraction. In keeping with this, we further reported that after interaction with ICAM-1, transendothelial migration invasion in MUC1 expressing cells is associated with increased MUC1-Src association, MUC1-cytoplasmic domain (MUC1-CD) phosphorylation, CrkL recruitment, and Rho-GTPase mediated cytoskeletal rearrangement [5-7].

MUC1 (also known as DF3, CA15-3, or episialin) is expressed apically on normal breast epithelia, but often loses this polarization and becomes underglycosylated in breast cancer [8,9]. MUC1 is translated as a single polypeptide, followed by conformational stress-induced cleavage resulting in a heterodimer of non-covalently associated extracellular and cytoplasmic portions [10,11] (Figure 1). The extracellular portion consists of a variable number of 20-amino acid (aa) tandem repeats containing multiple sites for O-glycosylation, which impart a negative charge and result in a structure that can extend up to 500 nm from the cell surface. The cytoplasmic portion consists of a 58-aa extracellular stub, a 28-aa transmembrane domain, and a 72-aa cytoplasmic domain, which contains seven conserved tyrosine residues, and has been shown to interact with diverse effectors [Reviewed in [12]] which is important since MUC1-CD itself lacks tyrosine kinase activity.

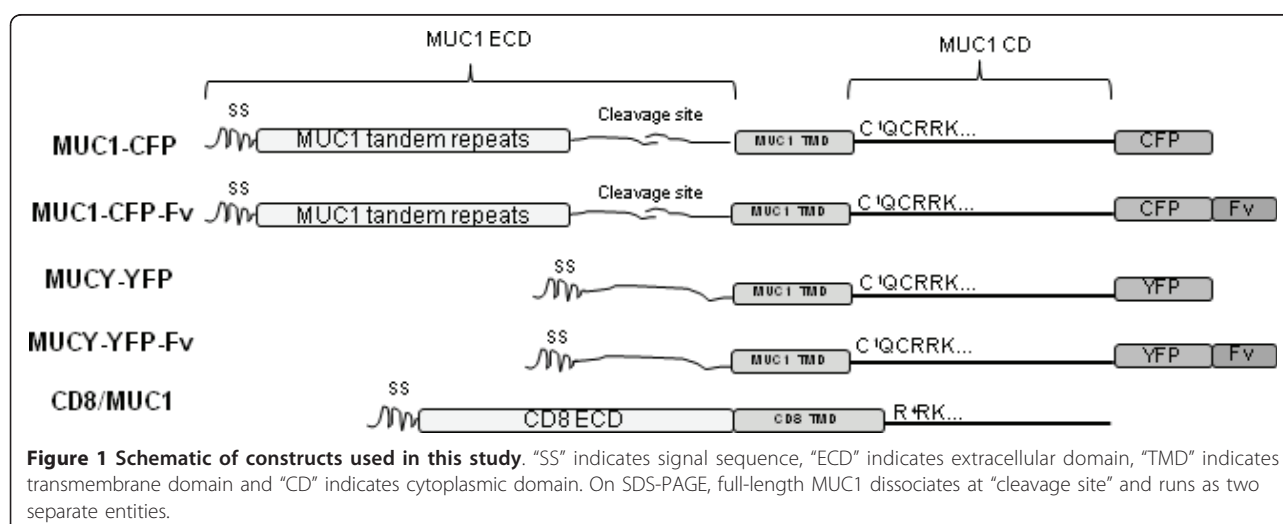
The signalling capacity of transmembrane proteins lacking kinase activity is often mediated by associated non-receptor tyrosine kinases. In some instances, these kinases are bound to pre-formed dimers of the receptor [[13], Reviewed in [14]]. Upon ligand binding, structural changes such as cysteine linkage, association with detergent resistant membrane fractions, and changes in cleavage result in signal initiation [15-17]. Previous work by others has demonstrated that constructs of the MUC1-CD form oligomers *in vitro* which are disulfide-linked,

and *in vivo* which are dependent on the membrane-proximal cytoplasmic C¹QC motif [18,19] (Figure 1). Here, we investigated dimer formation in wild-type MUC1 and the relationship between dimerization, Src recruitment and ICAM-1 induced signalling events. We also examined the role of membrane-proximal cytoplasmic domain cysteine residues in these phenomena. We confirm that Src is an essential mediator of the previously observed ICAM-1 binding pro-motility events and show that MUC1 forms constitutive cytoplasmic domain dimers which are required for constitutive Src recruitment and ICAM-1 binding induced signalling. Contrary to previous reports, we found that dimers are not disulfide linked constitutively or following ICAM-1 ligation, and that membrane-proximal cysteine residues are not required for dimerization or ICAM-1 induced events.

Materials and methods

Antibodies and Reagents

CT2 Armenian Hamster monoclonal antibody (mAb) [20], directed against the last 17 C-terminal amino acids of MUC1-CD, was generously provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). Rabbit anti-Src mAb, anti-Src^{P416} polyclonal Abs, and anti-rabbit peroxidase conjugated secondary antibody were purchased from Cell Signalling. Goat anti-mouse and anti-Armenian hamster peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. Mouse anti-tubulin antibody was from Sigma-Aldrich. Disuccinimidyl suberate (DSS) was from PierceNet. Protein G-Agarose was purchased from Roche Diagnostics. ECL Plus Western Blotting detection reagent was purchased from GE Healthcare (Amersham Biosciences). Gelatin Type A and phosphatase inhibitor cocktail were from Sigma-Aldrich. Protease inhibitor



cocktail was from Calbiochem. Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), Lipofectamine 2000, G418, Blasticidin S HCl, Pluronic F-127, Fluo-3 and Cell Tracker Green CMFDA were from Invitrogen. AP20187^D and AP21998^M were generous gifts from ARIAD Pharmaceuticals, Inc (Cambridge, MA, USA).

Plasmid construction

The pC1-Neo-hMUC1-TR+ plasmid was kindly provided by Dr. Sandra Gendler. The pUC-CVM-MUCY plasmid was from Gene-Therapeutics Luckenwalde (Luckenwalde, Germany). MUC1-CFP and MUCY-YFP were constructed by inserting the MUC1/MUCY genes into pECFP/pEYFP plasmids (ClonTech) respectively, at XhoI and SacII sites. The plasmid pC4-Fv1E encoding the FKBP F36V variant followed by a c-terminal hemagglutinin (HA) epitope was generously provided by ARIAD Pharmaceuticals Inc. To generate the MUC1-CFP-FvHA and MUCY-YFP-FvHA fusion proteins, the FvHA domain of pC4-Fv1E was amplified by polymerase chain reaction (PCR) with a 5' primer (ATTTGTA-CATGGCTTCTAGAGGAGTGC) and a 3' primer (CTCTTGTACACTGAAGTTCTCAGGATCC) which introduced 3' and 5' BsrGI restriction sites (underlined). The PCR product and MUC1-CFP/MUCY-YFP plasmids were digested with BsrGI, ligated, and sequenced to confirm insertion and orientation. MUC1-CFP-FvHA (CQC to AQA) was constructed by PCR of MUC1-CFP using overlapping forward (TTGGCTGTCTGCTCAGGCCCGCCGAAAG) and reverse (CTTTCGGCGGGCCTGAGCGACAGCCAA) primers to generate the mutation (underlined) and upstream (GGCACCTCTGCCAGGGCTACCACAACC) and downstream (GACCGGTGGATCCCGGGCCCG) primers containing EcoNI and BamHI restriction sites, respectively (underlined). Digestion of plasmid and PCR product with EcoNI and BamHI was followed by ligation of the plasmid backbone and mutated insert and sequencing to confirm insert. The pcDNA3.1-CD8/MUC1 plasmid was kindly provided by Dr. K.C. Kim (Lovelace Respiratory Research Institute, AZ), and encodes a construct containing the extracellular and transmembrane portions of cluster of differentiation 8 (CD8) and MUC1-CD, beginning at R⁴RK (Figure 1).

Cell culture

Human breast cancer cell lines T47D and MCF-7 were from the American Type Culture Collection (ATCC) and were maintained in DMEM with 10% FBS and 6 ug/ml insulin. 293T human embryonic kidney epithelial cells (293T HEK) were from ATCC and maintained in DMEM with 10% FBS. Mock and ICAM-1 transfected NIH 3T3 mouse fibroblast cells were a generous gift of

Dr. Ken Dimock (University of Ottawa, Ontario, Canada) and were maintained in DMEM with 10% FBS and 5 ug/ml Blasticidin S. HEK 293T cells transfected with MUC1 constructs were maintained in DMEM with 10% FBS and 200 ug/ml G418 and used for experiments within 48 hours of transfection. Cell lines have not been further tested or authenticated.

Small interfering ribonucleic acid (siRNA) knockdown

4×10^5 HEK 293T cells were plated in a 6-well plate and allowed to adhere overnight to approximately 50% confluency. siRNA (Dharmacon) consisted of four pooled siRNA species targeting the following Src sequences: GCAGUUGUAUGCUGUGGUU, GCAGAGAACCCGA GAGGGA, CCAAGGGCCUCA ACGUGAA, and GGGAGAACCUCUAGGCACA. Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Lipofectamine reagent only or non-targeting siRNA were used as negative controls.

Dimer detection

To detect constitutive dimers, we added a cross-linking agent before lysis to parallel cultures and analyzed by Western blot as follows. 3×10^6 human breast cancer cells or transfected HEK 293T cells were plated on 0.1% gelatin coated, UV-treated 10 cm dishes and allowed to adhere overnight. Cells were then serum starved for 45 minutes in Imaging Buffer (152 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂ 6H₂O, 1.8 mM CaCl₂ 2H₂O, 10 mM HEPES, 5.6 mM D-glucose). Treatment compounds or cell suspensions were then added as indicated, in 37°C Imaging buffer, followed by 1 mM DSS in ice-cold PBS for 10 minutes. DSS is a membrane-permeable, permanent crosslinker which targets primary amine residues (lysines) within 11.4 Å of each other. DSS does not induce dimerization of lysine-containing proteins, but rather aids in identification of protein complexes which are already formed upon treatment. DSS was aspirated, cells resuspended in quenching solution (1M Tris, pH 7.5) and centrifuged. The pellet was suspended in ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% glycerol, 1% Triton X-100, 0.5% phosphatase and protease inhibitors) or Co-immunoprecipitation lysis buffer (50 mM Tris pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5% protease and phosphatase inhibitors). Lysates were immunoprecipitated with CT2 and/or prepared for Western blot analysis as described below.

Dimer manipulation

To artificially manipulate MUC1-CD dimerization we used the "ARGENTTM Regulated homodimerization kit" and the "RPDTM Regulated secretion/aggregation kit"

(Ariad Pharmaceuticals, Inc.). The kits were designed to manipulate protein dimerization status by interacting an engineered "Fv dimerization domain" with monovalent and bivalent ligands. The "Fv domain" is a mutant of the naturally occurring FK506 binding protein (FKBP) with a F36V mutation introduced to prevent binding of Fv ligands to endogenous FKBP. A MUC1-CFP-Fv construct was created using this plasmid, as described above. Importantly, the Fv domain itself does not induce dimerization, and addition of Fv domain ligands is required to manipulate dimerization status of Fv domain containing proteins. The bivalent Fv ligand AP20187^D was designed to induce dimerization of Fv domain containing proteins, while the monovalent Fv ligand AP21998^M was designed to disaggregate existing dimers.

Immunoprecipitation and Western blots

Lysates were immunoprecipitated, prepared for SDS-PAGE, and probed for proteins of interest as described in [6]. Films were scanned with a Canon Canoscan 8600F, imported into Image J (NIH), contrast and brightness adjusted and cropped for presentation.

Calcium oscillation assay

Calcium oscillation assay was performed and analyzed as described [5]. Modifications included the treatment of cells with 1 μ M AP21998^M or AP20187^D for 1 minute prior to addition of NIH ICAM-1 cells, and the use of Eclipse software to obtain digital interference contrast (DIC) and fluorescent images.

Transwell migration assay

The upper membrane of Transwell inserts (Corning Costar, 6.5 mm diameter, 8 μ m pore size) coated with 0.1% gelatin and 200 μ l of ICAM-1/mock cell suspension at 1.5×10^5 cells/ml was placed in a 24-well plate and allowed to adhere overnight at 37°C. 293T MUC1 transfectants were suspended in 5 μ M Cell tracker green in serum-free DMEM for 30 minutes at 37°C, followed by incubation in serum free DMEM at 37°C for 30 minutes. Cells were spun and suspended in serum-free media at 8×10^5 cells/ml. 1 μ M AP21998^M or AP20187^D was added and 200 μ l of cell suspension was added to the upper membrane of Transwell inserts. Fresh serum-free DMEM was added to the lower chamber. Following incubation at 37°C overnight, media was removed and 2% paraformaldehyde in PBS was added to each chamber for 15 minutes. Cells were washed twice with PBS, and cells on the upper membrane of the insert were removed with a sterile cotton swab. The insert was then placed under a Zeiss Axioscope Digital Imaging Microscope and cells on the lower side of the chamber were counted under a fluorescein isothiocyanate filter and 20 \times objective for five distinct fields of view.

Statistics

All experiments were performed at least three times to allow for statistical analysis. The Newman-Keuls multiple range comparison was used to determine statistical differences in data sets with more than two experimental conditions. For pairwise comparisons, the Student's *t* test was used. P values are indicated for each analysis. For each experiment, pairs in the data set which are statistically different, or populations which do not overlap with any other in the data set, ($p < 0.05$) are indicated with an asterisk (*).

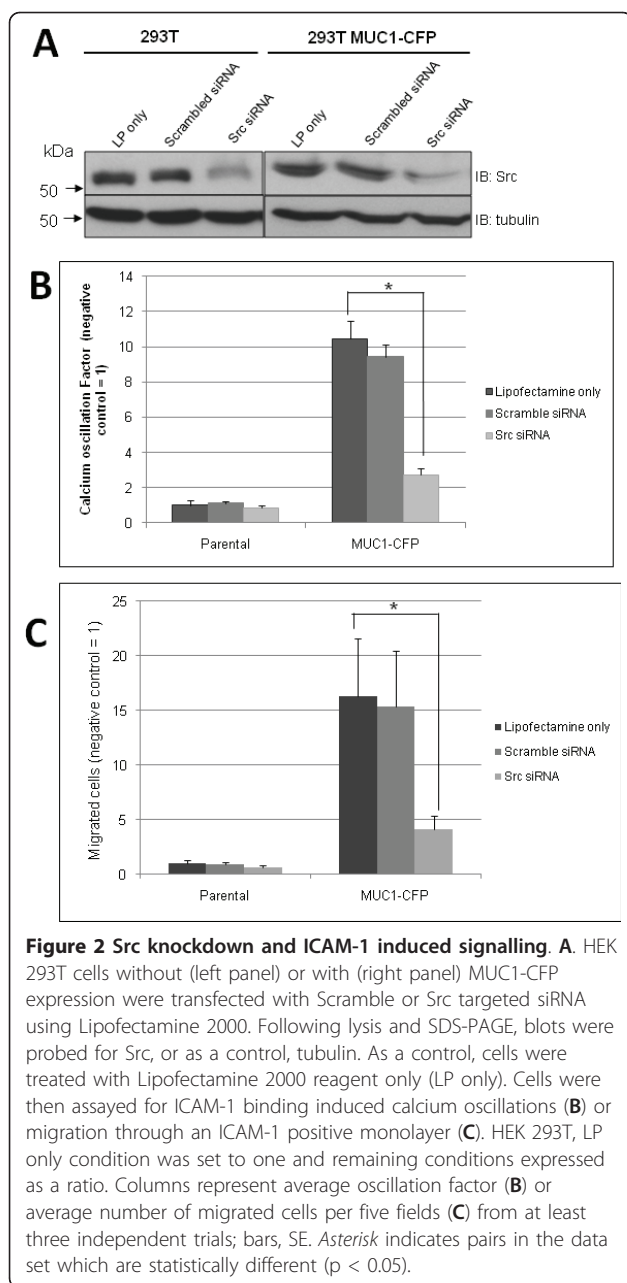
Results

MUC1/ICAM-1 binding induced signalling is mediated by Src kinase

We first confirmed that Src kinase is a critical component of the MUC1/ICAM-1 signalling axis by siRNA knockdown of Src in MUC1-CFP transfected HEK 293T cells. After Src siRNA treatment, we obtained a ~50% reduction in the levels of Src protein, compared to treatment with Lipofectamine alone or scrambled siRNA (Figure 2a). We then assayed for calcium oscillations (Figure 2b) and migration (Figure 2c), and found that MUC1-CFP cells treated with Lipofectamine-only respond to ICAM-1 stimulation by generating calcium oscillations (Figure 2b) and cell migration (Figure 2c), indicating that the presence of the CFP tail does not interfere with this response. Non-transfected HEK 293T cells which have no MUC1 showed no difference in ICAM-1 binding induced calcium oscillations or migration with decreased Src. Addition of scrambled siRNA to the transfected MUC1-CFP cells showed no decrease in Src levels and levels of ICAM-1 binding induced events equivalent to the Lipofectamine-only condition (negative control). However, Src siRNA induced Src knockdown in HEK 293T cells transfected with MUC1-CFP resulted in significant decreases in the ICAM-1 binding initiated calcium oscillations (Figure 2b) and transmigration through an ICAM-1 monolayer (Figure 2c). This establishes Src kinase as an essential mediator of MUC1/ICAM-1 binding signalling and migration.

MUC1 forms constitutive cytoplasmic domain dimers in human breast cancer cell lines and transfected HEK 293T cells

MUC1 positive human breast cancer cell lines MCF-7 and T47D (Figure 3a) and HEK 293T cells transfected with MUC1-CFP (Figure 3b, panel 1) or the MUC1 splice variant lacking the tandem repeat domain MUCY-YFP-Fv (Figure 3b, panel 2) were lysed with or without prior treatment with the membrane permeable crosslinker DSS. No Fv ligands were added, so only constitutive dimers of the MUCY-YFP-Fv are detectable. DSS reacts with primary amine containing amino acids



within 11.4 Å distance to produce covalently bonded complexes. It is important to note that DSS only reacts with lysine residues which are within 11.4 Å of each other prior to DSS treatment; DSS itself does not induce complex formation. MUC1-CD contains a membrane-proximal lysine residue (R^4RK) which would be susceptible to DSS crosslinking. Western blotting and probing for MUC1-CD in the cells treated with DSS revealed the invariable appearance of a new species at exactly double the molecular weight of the monomeric cytoplasmic domain, consistent with the presence of a MUC1-CD homodimer. The appearance of MUC1-CD dimers in

MUCY-YFP-Fv transfectants indicates that the tandem repeat domain is not responsible for dimerization. This is not surprising due to the heavy glycosylation and negative charge of the tandem repeats. We then investigated the contribution of the MUC1 cytoplasmic domain to dimer formation. HEK 293T cells were co-transfected with MUCY-YFP-Fv and/or CD8/MUC1 [21], a chimera of CD8 extracellular and transmembrane domains and MUC1-CD domain, beginning at R^4RK (Figure 1). Whole cell lysate of CD8/MUC1 (Figure 3c, lane 1) shows that this construct appears as a doublet at approximately 40 kDa in agreement with a publication describing this construct [21]. MUCY-YFP-Fv runs at approximately 75 kDa (Figure 3c, lane 2), with another species migrating at approximately 45 kDa. This species could be the result of cleavage of the YFP-Fv tag prior to cell lysis, as MUCY is expected to migrate at this molecular weight. Dual transfection of CD8/MUC1 and MUCY-YFP-Fv demonstrates that both these constructs run at the expected molecular weights when co-expressed (Figure 3c, lane 3). Immunoprecipitation of double transfectants with anti-CD8 resulted in the appearance of MUCY-YFP-Fv on a Western blot (Figure 3c, lane 4), indicating an association between CD8/MUC1 and MUCY-YFP-Fv. This association is significant because the CD8/MUC1 construct only contains the cytoplasmic portion of MUC1, beginning at R^4RK and does not contain the membrane proximal C^1QC motif, fluorescent tags or an Fv domain. Therefore association between these two entities must be due to the MUC1 cytoplasmic domain. “Protein G + Antibody” and “Antibody only” lanes are included to identify non-specific immunoglobulin bands. Taken together, these data indicate that the cytoplasmic domain of MUC1 self-associates to form a constitutive homodimer.

MUC1-CD dimerization is independent of membrane-proximal cysteine residues

Previous publications investigating MUC1 dimerization have concluded that the membrane-proximal CQC motif is responsible for disulfide-linked oligomerization, which results in targeting of MUC1 to the nucleus [18], and MUC1 mediated resistance to oxidative stress [19,22,23]. Since the CD8/MUC1-MUCY co-immunoprecipitation experiments (Figure 3c) found MUC1-CD association in the absence of a CQC motif in the CD8/MUC1 partner, we sought to determine if CQC mediated dimerization was necessary for our observed constitutive MUC1 dimers in MUC1 full-length transfectants and breast cancer cell lines. Using non-reducing conditions, which would preserve any disulfide linkages in Western blotting, there were no bands at a molecular weight of presumed dimers in 293T MUC1-CFP or MCF-7 cells (Figure 4a). This suggests that MUC1-CD

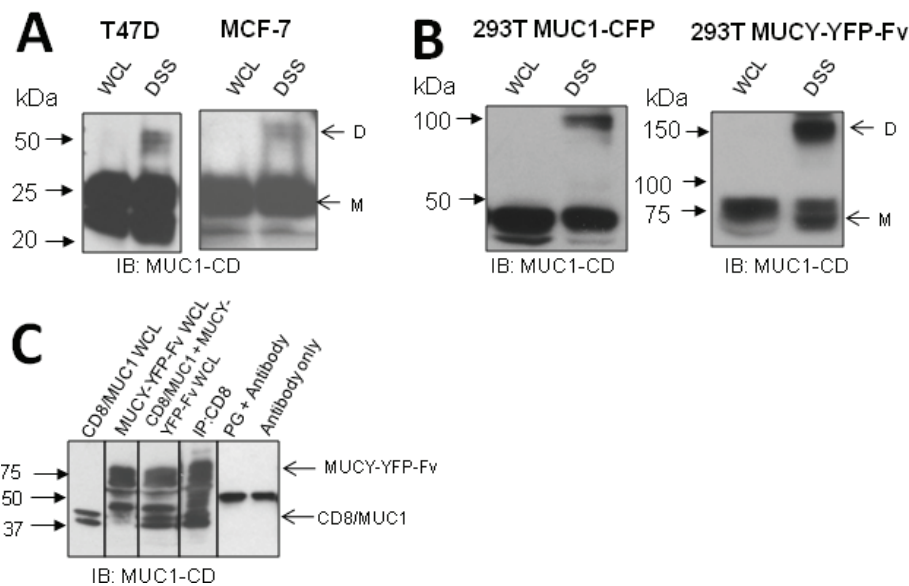


Figure 3 MUC1 dimerization in human breast cancer and transfected cell lines. **A.** Breast cancer cell lines T47D and MCF-7, or **(B)** HEK 293T cells transfected with MUC1 constructs MUC1-CFP and MUCY-YFP-Fv were treated with DSS or no treatment control, lysed, ran on SDS-PAGE and probed with anti-MUC1-CD. **C.** HEK 293T cells were transfected with CD8/MUC1 (lane 1) MUCY-YFP-Fv (lane 2), or both (lane 3), lysed, and double transfectants immunoprecipitated with anti-CD8 (lane 4). Lysates were ran on SDS-PAGE and probed with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates (WCL), PG (Protein G) + Antibody (anti-CD8) and Antibody only (Anti-CD8) lanes are included as controls.

dimers are not disulfide linked. We confirmed this by mutating both cysteines in the native MUC1-CD CQC motif to alanines (AQA) and then assaying for dimers after DSS cross-linking. Note that no Fv ligands were added so that any dimers detected represent constitutive or pre-formed dimers. We found that the 293T MUC1-CFP-Fv (AQA) mutant formed cytoplasmic domain dimers (Figure 4b). The presence of DSS-stabilized dimers in the absence of Fv ligands indicates that constitutive MUC1-CD dimers form even when both

cysteines are absent and constitutes conclusive proof that cellular MUC1-CD dimers are not disulfide linked.

MUC1 cytoplasmic domain dimerization can be disrupted by an engineered Fv domain and a monomeric ligand

To investigate the importance of MUC1 dimerization in Src association and ICAM-1 induced signalling, we manipulated dimerization using a chimeric construct of MUC1 and a C-terminal Fv domain (ARIAD Pharmaceuticals), which is FKBP (FK506 binding protein) with a F36V mutation, allowing for specific interaction between the engineered Fv domain and bivalent (AP20187^D) or monovalent (AP21998^M) ligands. Importantly, the Fv domain itself does not facilitate dimerization of proteins, but following addition of Fv domain ligands, dimerization status can be manipulated. Previously, this system has been used to successfully manipulate dimerization of growth factor receptors [24] and G protein-coupled receptors [25]. Mechanistically, the bivalent ligand, which contains two Fv-binding domains, effectively brings two Fv-domain containing proteins within close proximity - "dimerization". The monovalent ligand, which contains one Fv-domain binding domain, is designed to bind to Fv-domain containing proteins and sterically inhibit their interaction with other proteins - "disaggregation" or "monomerization" (Figure 5a). MUC1-CD dimers were stabilized after Fv ligand treatment by addition of the DSS cross-linker prior to

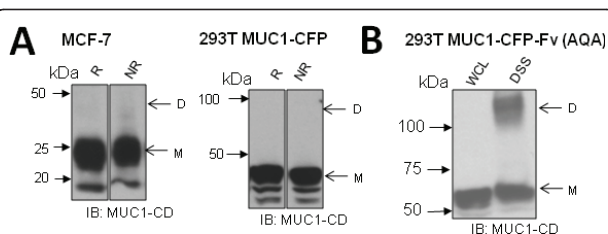


Figure 4 Covalent bonds and cysteine residues in MUC1 dimerization. **A.** Breast cancer cell line MCF-7 or HEK 293T cells transfected with MUC1-CFP were subjected to reducing ("R"; β -mercaptoethanol added) or non-reducing ("NR"; no β -mercaptoethanol) conditions, ran on separate SDS-PAGE to prevent diffusion of β -mercaptoethanol, and probed with anti-MUC1-CD. **B.** 293T MUC1-CFP-Fv AQA cells were treated with DSS, lysed, ran on SDS-PAGE, and probed with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates (WCL) are included as controls.

cell lysis. We found that treatment of 293T MUC1-CFP-Fv cells for one minute with increasing concentrations of AP20187^D did not increase the quantity of MUC1-CD dimers above no treatment, while AP21998^M treatment resulted in a dose dependant reduction in MUC1-CD dimerization (Figure 5b). After treatment with 1 μ M of monomerizing Fv ligand, AP21998^M there was a 60% reduction in detectable MUC1-CD dimers (Figure 5c). As a control, 293T MUC1-CFP cells, which lack the Fv domain, do not show a change in dimer quantity following treatment with AP20187^D or AP21998^M (Figure 5d). Densitometric analysis of the MUC1-CFP dimer band normalized to monomer band illustrates this observation further (Figure 5e).

MUC1-CD dimer disruption results in decreased recruitment of total and active Src kinase

To determine the importance of MUC1-CD dimerization in constitutive Src recruitment, 293T MUC1-CFP-Fv (Figure 6a, b), and, as a control, 293T MUC1-CFP (Figure 6c, d) cells were treated with increasing concentrations of AP20187^D or AP21998^M for one minute and immunoprecipitated with anti-MUC1-CD. Following separation on SDS-PAGE, blots were probed with anti-Src (total) and anti-Src^{P416} (active). In the MUC1-CFP-Fv transfectants, the amount of total and active Src associated with MUC1-CD decreases in a dose-dependent manner with AP21998^M treatment (Figure 6b, arrows). Treatment with AP20187^D did not result in a significant change, and 293T MUC1-CFP cells were unaffected by Fv ligand treatment.

Densitometric analysis of Src and Src^{P416} compared to MUC1-CD are given in Additional File 1. These data suggest that MUC1-CD dimers, but not monomers, contain a recruitment, and potentially an activation, motif for Src kinase.

MUC1-CD dimer disruption results in decreased ICAM-1 binding induced calcium oscillations and cell invasion

To determine if MUC1-CD dimerization is important in previously observed ICAM-1 binding induced events, we assayed parental (293T), 293T MUC1-CFP, and 293T MUC1-CFP-Fv, and 293T MUC1-CFP-Fv (AQA) cells for ICAM-1 binding induced calcium oscillations, and invasion through an ICAM-1 positive monolayer after addition of the Fv ligands 1 μ M AP20187^D or 1 μ M AP21998^M and compared this to a no treatment control. 293T MUC1-CFP and 293T MUC1-CFP-Fv cells responded to treatment with ICAM-1 positive cells, in the "no treatment" condition, by initiating calcium oscillations (Figure 7a) and increased invasion (Figure 7b) at levels which were significantly increased compared to Mock treatment and statistically equivalent, demonstrating that the addition of the CFP or CFP-Fv tag on the

C-terminus did not affect MUC1 receptor response to ICAM-1 stimulation. The 293T MUC1-CFP-Fv (AQA) mutant also exhibited ICAM-1 binding induced calcium oscillations (Figure 8a) and cell invasion (Figure 8b) equivalent to the native 293T MUC1-CFP-Fv cells indicating that the CQC motif is not required for ICAM-1 induced signalling events. However, ICAM-1 binding induced calcium oscillations (Figure 7a) and invasion (Figure 7b) in 293T MUC1-CFP-Fv cells was significantly reduced by treatment with AP21998^M, while prolonged treatment with AP20187^D resulted in a significant increase in cell migration in 293T MUC1-CFP-Fv cells (Figure 7b). These data indicate that MUC1-CD dimerization is required for ICAM-1 binding induced events. Addition of the Fv ligands had no significant effect on the 293T MUC1-CFP transfectants lacking the Fv domain. As previous reports [19] have demonstrated that disruption of dimerization using peptides results in cell death, we performed a trypan blue exclusion assay after treatment with 1 μ M AP20187^D or AP21998^M and saw no significant reduction in viability up to 72 hour exposure (See Additional File 2).

ICAM-1 ligation does not result in increased MUC1-CD dimerization or disulfide linkage of MUC1-CD dimers

Next, we investigated if ICAM-1 ligation results in a quantitative increase in MUC1 dimerization, a potential mechanism for signal initiation. T47D and 293T MUC1-CFP cells were treated with ICAM-1 transfected NIH 3T3 cells for 10 or 60 seconds prior to DSS treatment. These time points were chosen because previous work has demonstrated that increased Src and CrkL recruitment, MUC1-CD phosphorylation [6] and calcium oscillations [5] occur within one minute of ICAM-1 ligation. We found that ICAM-1 ligation did not increase the quantity of MUC1-CD dimer detected (Figure 9a), suggesting that a qualitative, rather than quantitative, change in MUC1-CD dimers is responsible for ICAM-1 induced signalling. We then considered the possibility that ICAM-1 binding triggers disulfide bridge formation, akin to growth hormone binding to pre-formed growth hormone receptor dimers [15], but were not able to detect MUC1-CD dimers in MCF-7 or 293T-MUC1-CFP cells subjected to reducing or non-reducing conditions after ICAM-1 treatment for 60 seconds (Figure 9b). As a control, the CD8/MUC1 chimera, which is disulfide linked via the CD8 extracellular region [26,27], was run under reducing and non-reducing conditions. The appearance of a disulfide-linked dimer under non-reducing conditions (Figure 9c) validates our methods.

Discussion

The MUC1 glycoprotein has been implicated in multiple tumorigenic processes including tumour formation,

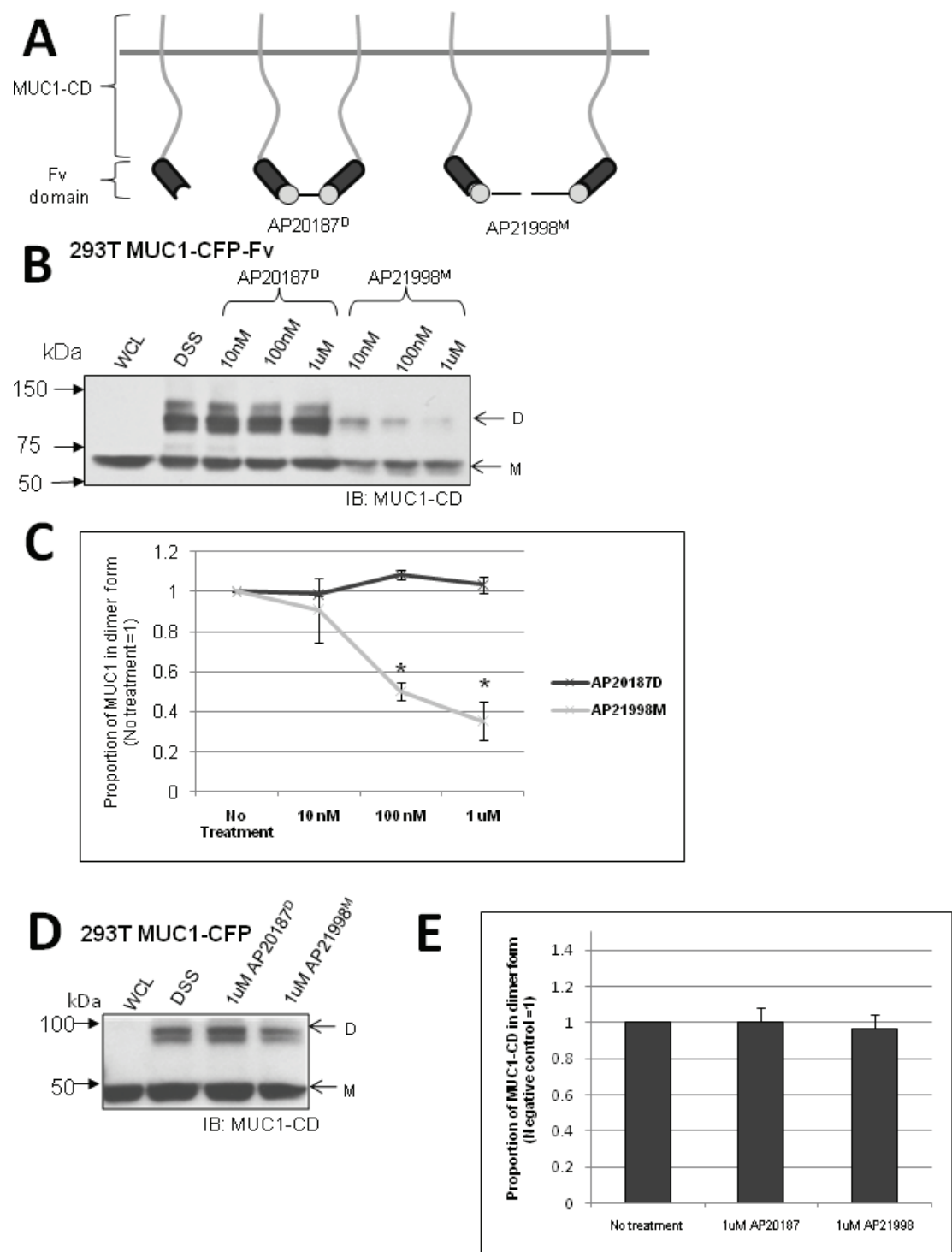


Figure 5 Manipulation of MUC1 dimerization using an engineered domain. **A.** Schematic of the mechanism of dimer formation/disruption by Fv ligands. **B.** Treatment of 293T MUC1-CFP-Fv cells with AP20187^D/AP21998^M and DSS. **C.** Densitometric analysis of dimer bands from **B** normalized to total MUC1. "No treatment" control refers to "DSS" only treatment from (**B**), and it set to one with the remaining conditions expressed as a ratio. **D.** Treatment of 293T MUC1-CFP cells with AP20187^D/AP21998^M and DSS. **E.** Densitometry of MUC1-CFP dimer bands from **D** normalized to total MUC1. "No treatment" control refers to "DSS" only treatment from (**D**) and is set to one with the remaining conditions expressed as a ratio. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates (WCL) are included as controls. Asterisk indicates a discrete population that does not overlap with any other population in the data set, $p < 0.05$.

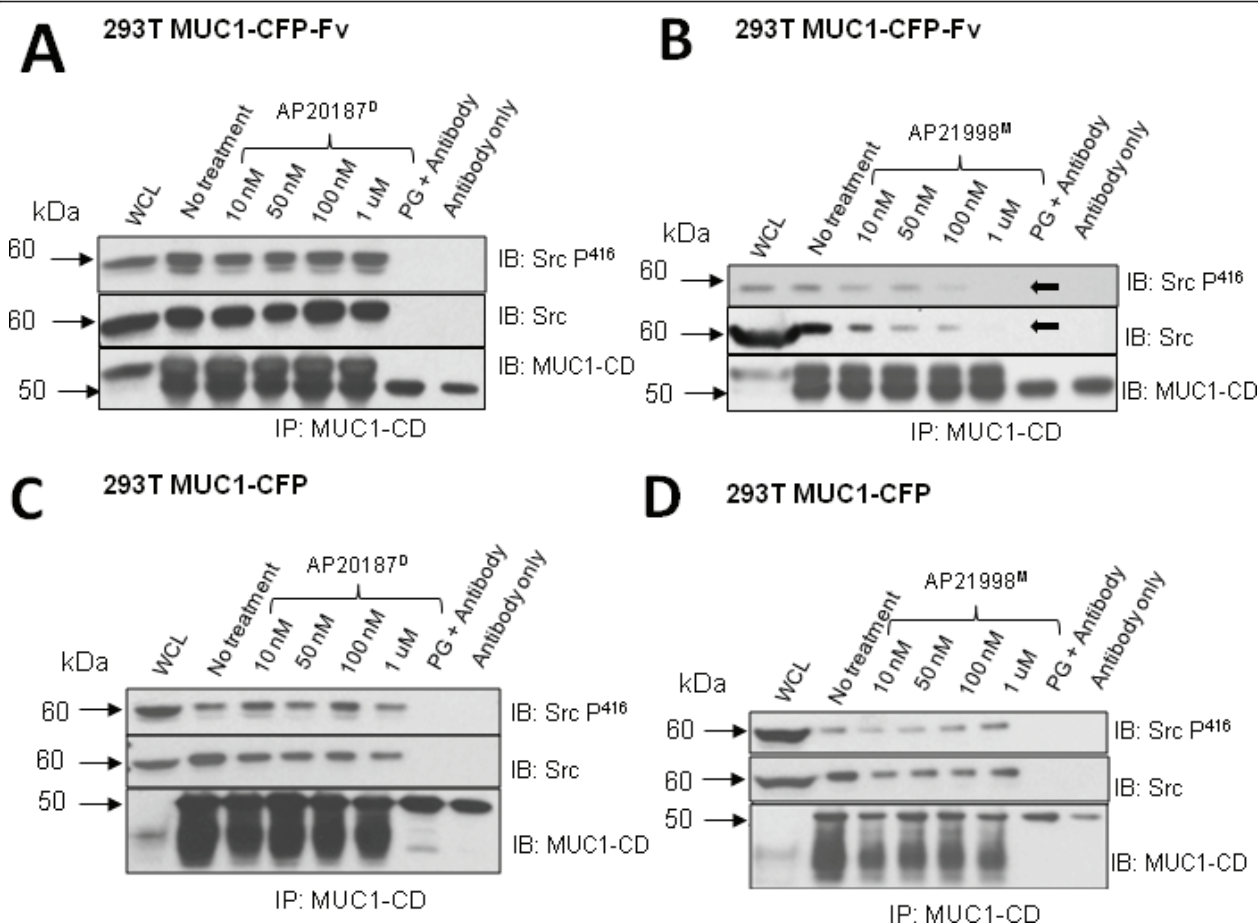
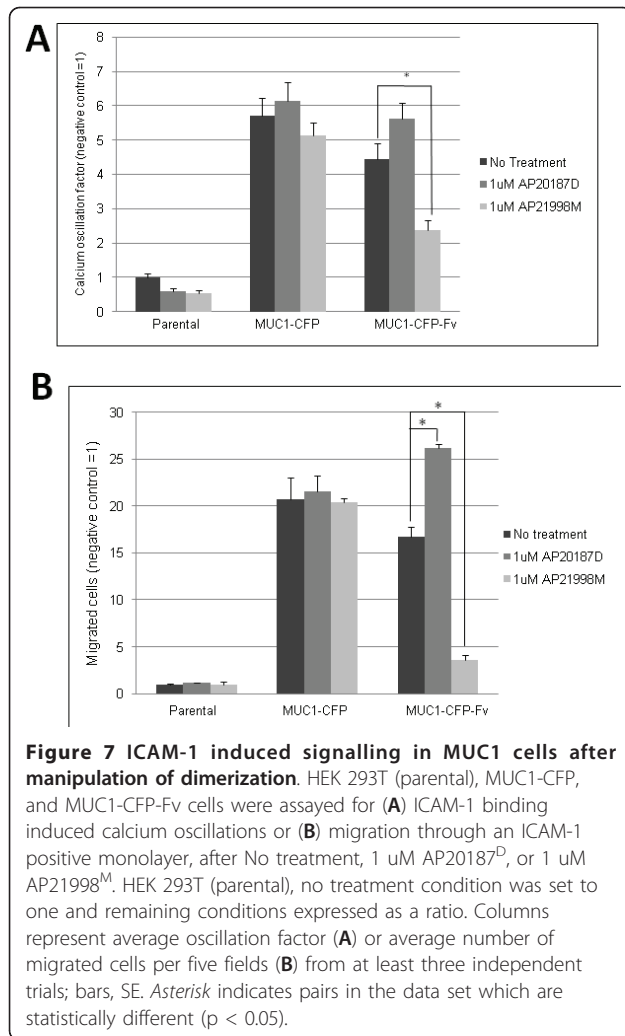


Figure 6 Src and Src^{P416} recruitment to MUC1 after manipulation of dimerization. Co-immunoprecipitation with anti-MUC1-CD was performed on 293T MUC1-CFP-Fv cells (**A, B**) and MUC1-CFP cells (**C, D**) after treatment with increasing concentrations of AP20187^D (**A, C**) or AP21998^M (**B, D**). Immunoprecipitates were probed for Src^{P416}, Src, and anti-MUC1-CD (as a loading control) with stripping of blots between each probe. Whole cell lysates (WCL), PG (Protein G) + Antibody and Antibody only lanes are included as controls. Densitometric analysis of Src and Src^{P416} compared to MUC1-CD is presented in Additional File 1.

proliferation, and survival [19,22,28,29]. We are unique in investigating the role of MUC1 in the motility of Luminal B breast cancer cell lines, focusing on the binding of MUC1 to ICAM-1. ICAM-1 is expressed throughout the migratory track of a metastasizing breast cancer cell and its role in leukocyte extravasation is well characterized [30,31]. Although we have previously shown that MUC1/ICAM-1 ligation induces pro-motility behaviour [5-7], the mechanism of signal initiation was unknown. In this report, we show that dimerization of the MUC1-CD is essential for the ICAM-1 induced events and that this effect is most likely mediated through enhanced Src binding.

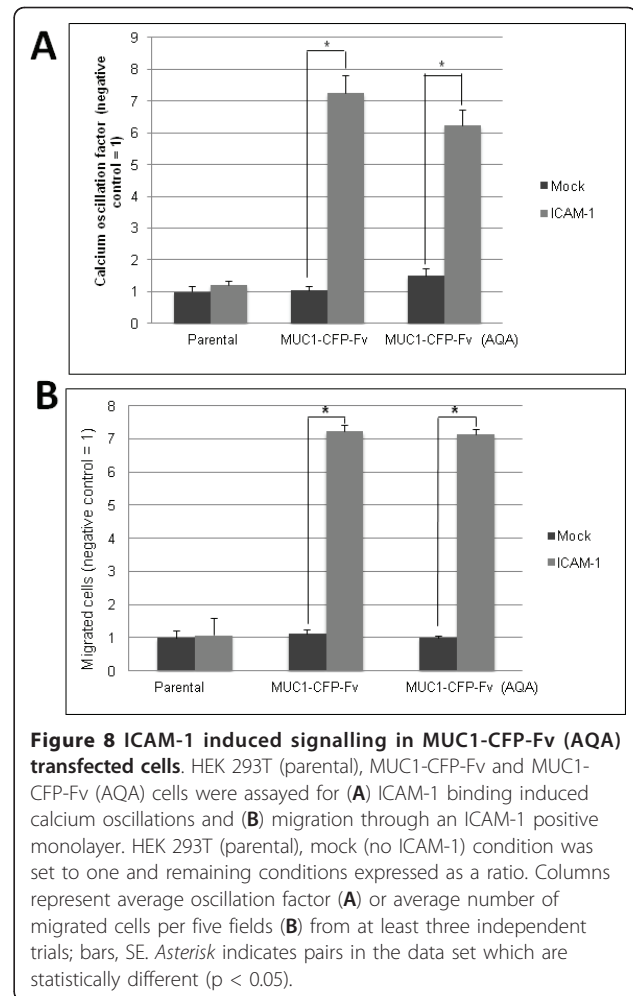
The signalling capacity of transmembrane proteins lacking kinase activity is often mediated by associated non-receptor tyrosine kinases. Here we show that Src kinase is essential for transmission of the migration related MUC1/ICAM-1 signal. This is consistent with

the literature on Src inhibition in breast cancer. Even though transfection of Src alone does not have transforming ability [32], over activity of Src is commonly associated with breast tumour progression [33] and it has become a prime target for selective small molecule inhibitors: Dasatinib (Bristol-Myers Squibb), Bosutinib (Wyeth) and Saracatinib (AstraZeneca). Others have published that the MCF-7 luminal B cell line used in this study shows decreased migration when Src is inhibited [34-36]. This decrease in Src mediated cell migration is synergistic with concomitant Tamoxifen [37], associated with upregulation and stabilization of E-cadherin/ β -catenin mediated intercellular adhesion [36,38], and decreased activity of the integrin associated kinase, focal adhesion kinase (FAK) [34,37]. These observations are consistent with MUC1 involvement in the observed Src motility pathway. Tamoxifen decreases MUC1 expression [39,40] and down-regulation of MUC1 is



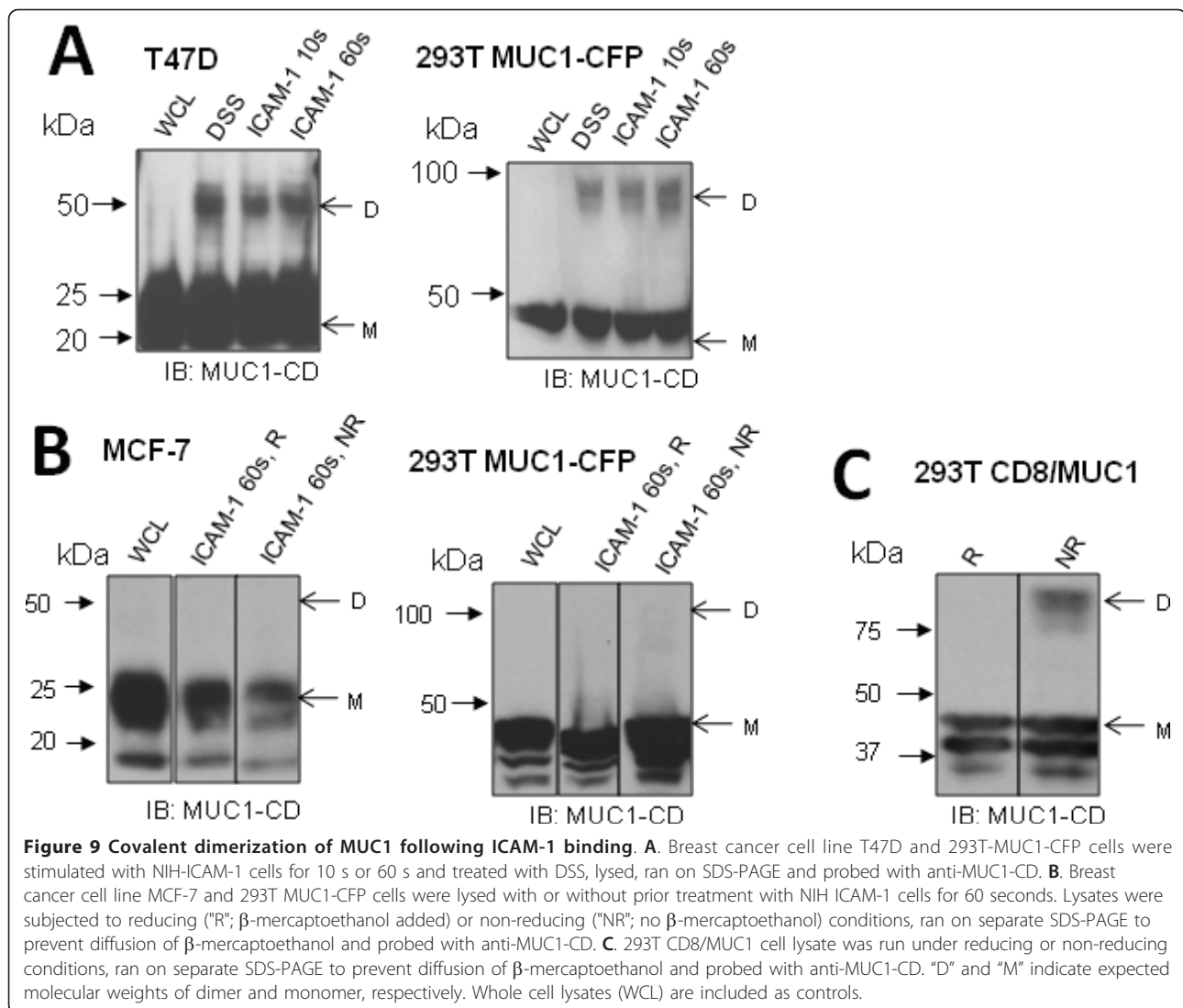
associated with increased E-cadherin/ β -catenin complex formation [41]. Further, MUC1 has been shown to bind to FAK, possibly transporting Src to FAK forming a MUC1-Src-FAK complex, and increasing FAK activation [28]. Thus the additive effect of Tamoxifen, the stabilization of intercellular adhesions and the decreased FAK activity are logical consequences of the dual inhibition of MUC1 and Src in the same pathway.

The association between MUC1 and Src is dependent on the existence of MUC1-CD dimers, indicating that MUC1-CD dimers adopt a conformation that is permissive Src binding. We have definitive (unpublished) data demonstrating that a Src-SH3 peptide binds to MUC1 constitutively via the putative SH3 binding domain R³⁴XXP³⁷P³⁸XXXXR⁴³. Binding of the Src SH3 domain has been previously described as a mechanism for partial unfolding of the inactive Src enzyme and can be associated with Src activation [42] suggesting a mechanism for MUC1-CD dimer activation of Src. In this regard, it is significant that Src-P⁴¹⁶, which is indicative



of fully active Src, also selectively binds to MUC1-CD dimers.

Classically, it was believed that surface membrane receptors existed as monomers until ligand binding induced dimerization of the receptors, allowing trans-activation of receptor associated kinases and the triggering of signal initiating phosphorylation cascades. In recent years, a new paradigm, typified by the growth hormone receptor (GHR), has emerged in which receptors exist as pre-formed ligand-independent dimers [13], Reviewed in [14]. Upon ligand binding to the dimers, structural changes such as cysteine linkage, association with detergent resistant membrane fractions or changes in receptor cleavage result in signal initiation [15-17]. We report here that the MUC1 cytoplasmic domain exists constitutively as a non-covalently linked dimer. We present evidence that in the absence of the transmembrane and extracellular domains, the cytoplasmic domain of MUC1 self-associates in a non-cysteine dependent fashion. It has been proposed that a “self-aggregation domain” exists in the extracellular stub of



MUC1-CD [43], but further studies are required to address the possibility that functional dimerization in vivo involves several domains.

In this study, membrane proximal cysteine residues are not required for dimerization of MUC1-CD, Src recruitment, or ICAM-1 induced signalling indicating that disulfide bridge formation is not the ligand-induced signal initiating event, as has been proposed for GHR. This is to be expected since, in the reducing environment of the cytosol, formation and maintenance of disulfide bonds is unfavourable unless the redox balance is disrupted [44]. Thus, disulfide linkage of MUC1 dimers reported by others [18,19], represents an alternative functional pathway for MUC1-CD dimers, perhaps as a redox "sensor" [Reviewed in [45]], that is unrelated to the ICAM-1/Src motility pathway in the present study. In this way, cysteine-mediated dimerization of MUC1 in response to oxidative stress, could initiate a signalling

cascade resulting in the demonstrated nuclear entry and expression of anti-oxidant enzymes ascribed to cysteine-linked MUC1-CD dimers [19,22,23].

Rational drug combination has received considerable interest in recent years [Reviewed in [46]] as it provides the opportunity for specific, synergistic inhibition of cell signalling pathways. Initial clinical results using Src inhibitors as single agents has shown them to be well-tolerated but have minimal anti-tumour response in patients [47]. Several Src inhibitors are currently undergoing testing in clinical trials for use in breast cancer treatment alone and in combination with other inhibitors [Reviewed in [48]]. The subset of luminal B cancers with active Src kinase pathway [49] may be the ideal target for a combined Tamoxifen and anti-Src therapy. Our studies suggest that if these could be combined with an inhibitor of MUC1 dimerization that cell migration and metastases may be significantly

decreased, possibly without the toxic effects of classic chemotherapy.

Conclusion

The MUC1 protein is overexpressed in the majority of breast cancers and is implicated in breast cancer metastasis. We show here for the first time that MUC1-CD forms non-covalently linked dimers which are required for recruitment of Src kinase, and ICAM-1 induced pro-metastatic events. This is significant because ICAM-1 is expressed throughout the migratory tract of a metastasizing breast cancer cell. Ligation of MUC1 and ICAM-1 may represent a mechanism for movement of breast cancer cells through stromal and endothelial tissues. Therefore, elucidation of the mechanism of MUC1/ICAM-1 signalling will reveal potential targets for anti-metastatic therapies. Our study sheds light on this mechanism and also demonstrates the need for additional research to resolve discrepancies in the field.

Additional material

Additional file 1: Densitometry of Src and Src^{P416} bands from SDS-PAGE (Figure 6) normalized to MUC1-CD. Using ImageJ software (NIH), the Src and Src^{P416} bands were analyzed for densitometric intensity, and values were normalized to the intensity of the corresponding MUC1-CD band to control for protein loading. The values were then graphed versus treatment and dose for each cell line.

Additional file 2: Growth curve of MUC1-CFP-Fv cells after treatment with AP20187^D or AP21998^M. Using Trypan blue exclusion assay, the number of live cells in a sample were counted daily for 3 days. The number of live cells in the sample was then extrapolated to estimate live cells in the population. No significant difference was found in the populations treated with AP21087^D, AP21998^M, or no treatment control.

List of Abbreviations

Å: angstrom; aa: amino acid; Ab: antibody; ATCC: American tissue culture collection; CD8: cluster of differentiation 8; DIC: digital interference contrast; DMEM: Dulbecco's modified eagle media; DSS: disuccinimidyl suberate; FAK: focal adhesion kinase; FBS: fetal bovine serum; FKBP: FK506 binding protein; GHR: growth hormone receptor; HA: hemagglutinin; HEK: human embryonic kidney; ICAM-1: Intercellular adhesion molecule-1; MUC1: Mucin-1; MUC1-CD: Mucin-1 cytoplasmic domain; PBS: phosphate buffered saline; PCR: polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; siRNA: small interfering ribonucleic acid.

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Author details

¹Department of Laboratory Medicine and Pathology, 3-70 Heritage Medical Research Centre, University of Alberta, Edmonton, AB, T6G 2S2, Canada.

²Department of Pediatrics, University of Maryland, School of Medicine,

Baltimore, MD, 21201, USA. ³Department of Oncology, Cross Cancer Institute, University of Alberta, Edmonton, AB, T6G 1Z2, Canada.

Authors' contributions

AB contributed to study design, performed all experiments, analyzed data, contributed to data interpretation, and drafted the manuscript. JZ contributed to study design and constructed plasmids. EL contributed the MUC1/CD8 plasmid and contributed to data interpretation. AS contributed to study design and data interpretation. NG performed complimentary studies that aided in the interpretation of this data. JH conceived the study, contributed to study design, and helped to draft and edit the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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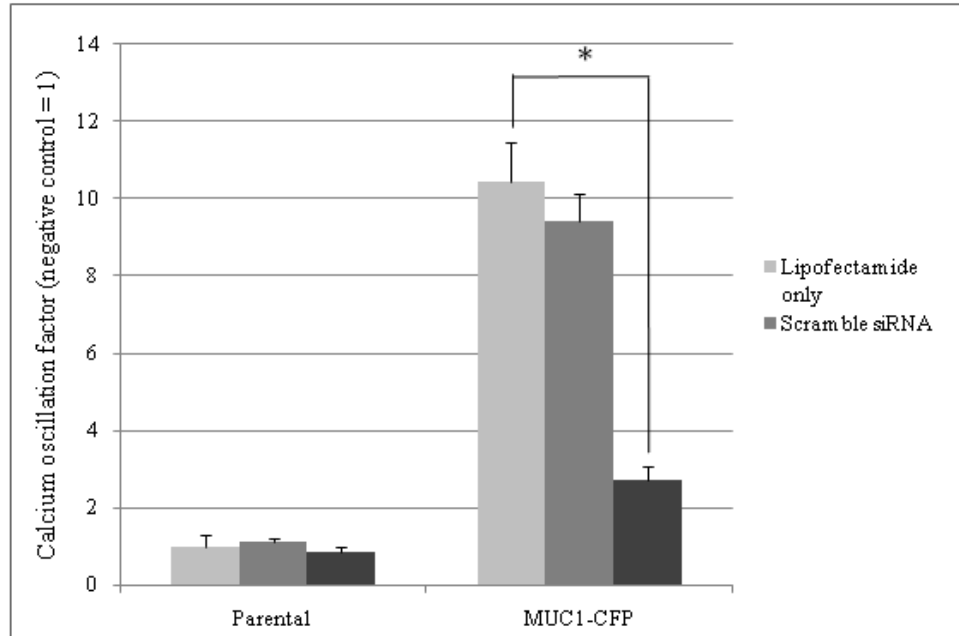


Figure 3.4. siRNA knockdown of Src inhibits ICAM-1 binding induced calcium oscillations in MUC1 expressing cells. HEK 293T (Parental) cells and MUC1-CFP transfectants were treated with NIH ICAM-1 cells and assayed for calcium oscillation factor. Lipofectamide only and Scramble siRNA treatments are included as controls. Parental Lipofectamide only condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average oscillation factors from at least three independent trials; bars, SE. Asterisk indicates pairs in the data set which are statistically different ($p < 0.05$).

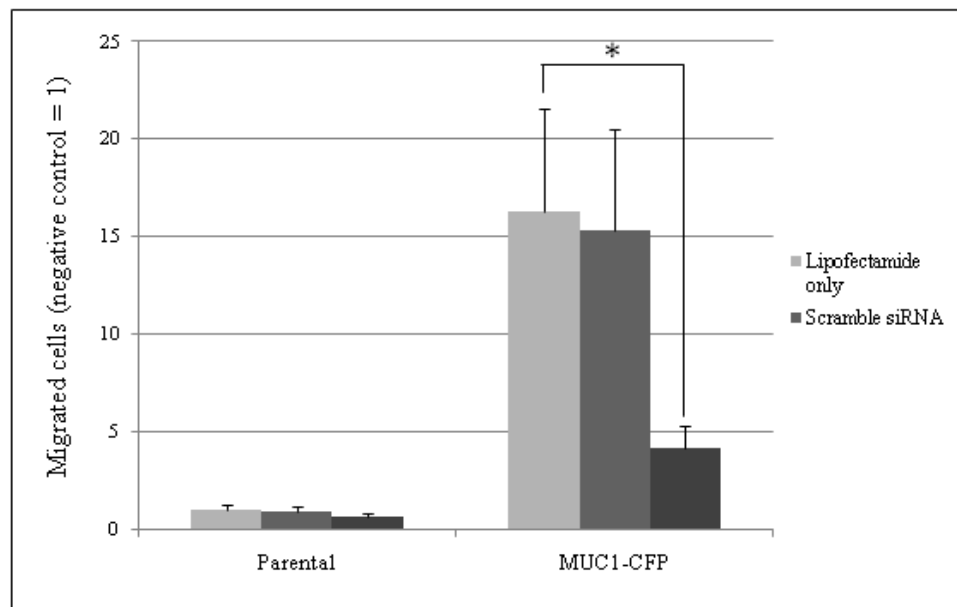


Figure 3.5. siRNA knockdown of Src inhibits ICAM-1 binding induced cell migration in MUC1 expressing cells. HEK 293T cells and MUC1-CFP transfectants were assayed for migration through an ICAM-1 positive cell monolayer. Lipofectamide only and Scrambled siRNA treatments are included as controls, and 293T Lipofectamide only condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average number of migrated cells per five fields from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p < 0.05$).

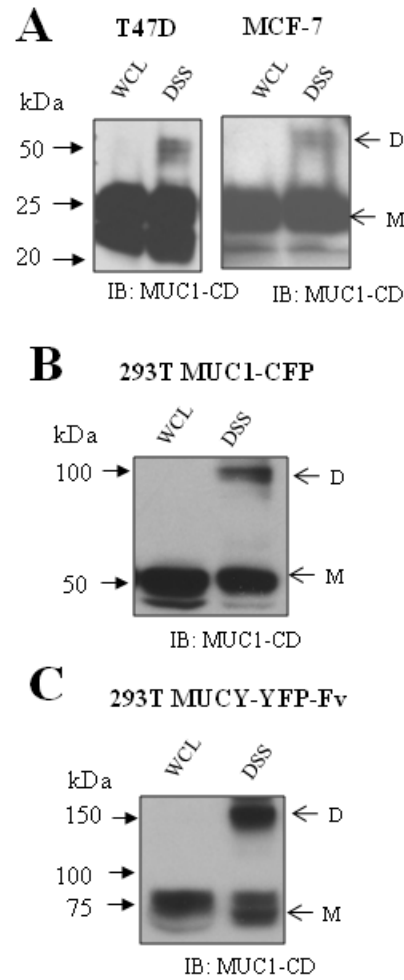


Figure 4.1. MUC1 dimerization in human breast cancer and transfected cell lines. A. Breast cancer cell lines T47D and MCF-7, or HEK 293T cells transfected with MUC1 constructs MUC1-CFP (B) or MUCY-YFP-Fv (C) were treated with DSS, lysed, ran on SDS-PAGE and probed with anti-MUC1-CD. “D” and “M” indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysate (WCL) lanes are included as controls.

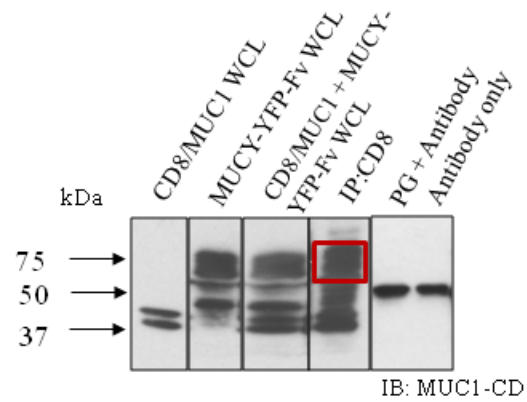
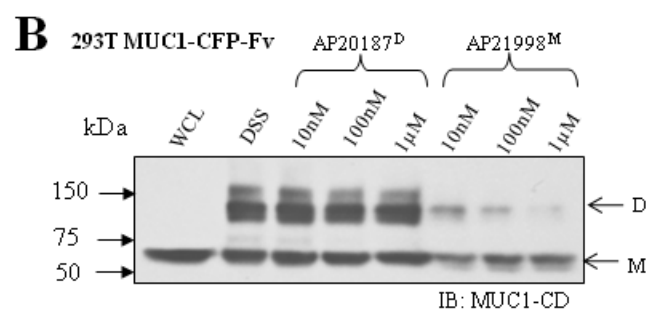
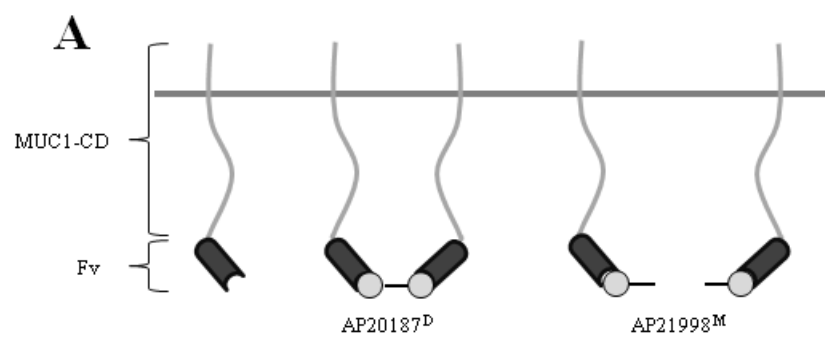


Figure 4.2. The cytoplasmic domain of MUC1 self-associates *in vivo*. HEK 293T cells were transfected with CD8/MUC1 (Lane 1), MUCY-YFP-Fv (Lane 2), or both (Lane 3), lysed, and double transfectants immunoprecipitated with anti-CD8 (Lane 4). Lysates were run on SDS-PAGE and probed with anti-MUC1-CD. PG (Protein G) + Antibody and Antibody only lanes are included as controls. Immunoprecipitation of MUCY-YFP-Fv with anti-CD8 is indicated by the red square.



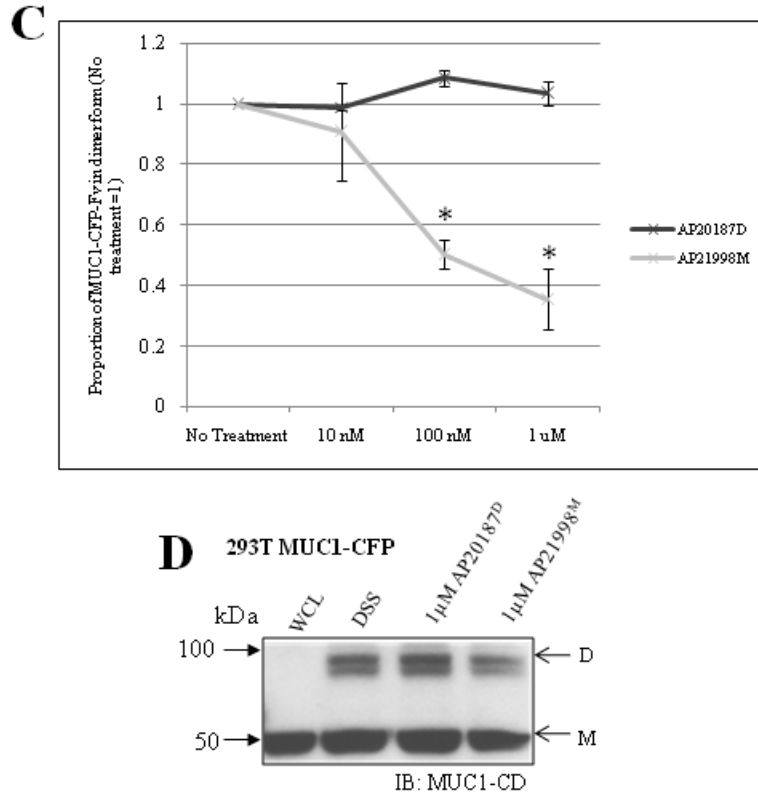


Figure 4.3. Dimerization of MUC1 can be manipulated by addition of an Fv domain and divalent and monovalent Fv domain ligands. A. Schematic of the mechanism of dimer formation/disruption by Fv ligands. B. Treatment of 293T MUC1-CFP-Fv cells with AP20187^D/AP21998^M and DSS, followed by SDS-PAGE and probing with anti-MUC1-CD. C. Densitometric analysis of dimer bands from (B) normalized to total MUC1-CD reveal proportion of MUC1-CD in dimer form. “No treatment” condition is set to one and remaining conditions expressed as a ratio. D. Treatment of 293T MUC1-CFP cells with AP20187^D/AP21998^M and DSS, followed by SDS-PAGE and probing with anti-MUC1-CD. “D” and “M” indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates (WCL) are included as controls. *Asterisk* indicates a discrete population that does not overlap with any other population in the data set ($p < 0.05$).

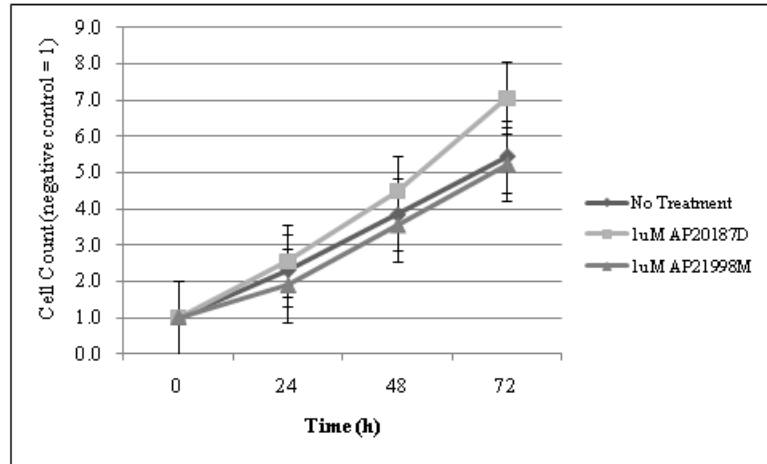


Figure 4.4. Treatment of MUC1-CFP-Fv cells with AP20187^D and AP21998^M does not affect cell viability. Following treatment for 24, 48, or 72 hours with 1 μM AP20187^D or AP21998^M, or no treatment control, cells were assayed for viability using the trypan blue exclusion assay. Cell count prior to experimentation was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates conditions which do not overlap ($p < 0.05$) with any other population in the data set.

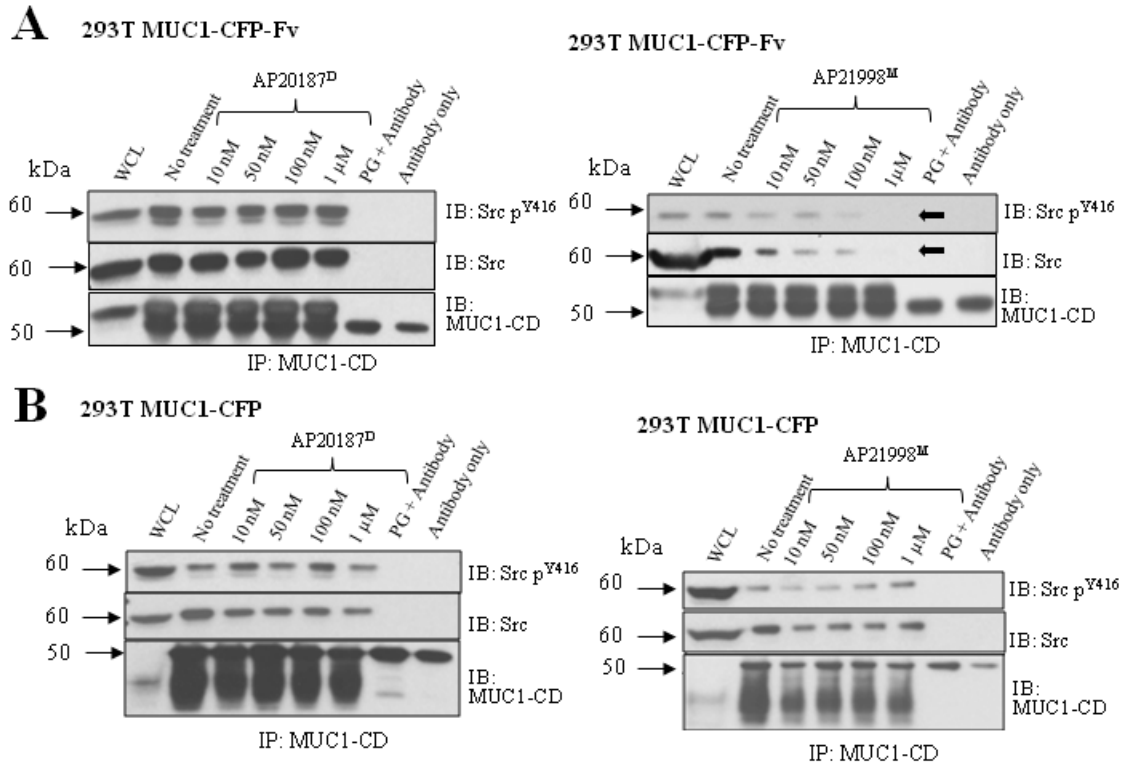


Figure 4.5. Recruitment of Src and Src p^{Y416} to MUC1-CD can be disrupted by inhibition of MUC1-CD dimerization. Co-immunoprecipitation with anti-MUC1-CD was performed on 293T MUC1-CFP-Fv cells (A) and MUC1-CFP cells (B) after treatment with increasing concentrations of AP20187^D (left panels) or AP21998^M (right panels). Immunoprecipitates were probed for Src p^{Y416}, Src, and MUC1-CD (as a loading control) with stripping of blots between each probe. Whole cell lysate (WCL), PG (Protein G) + Antibody and Antibody only lanes are included as controls.

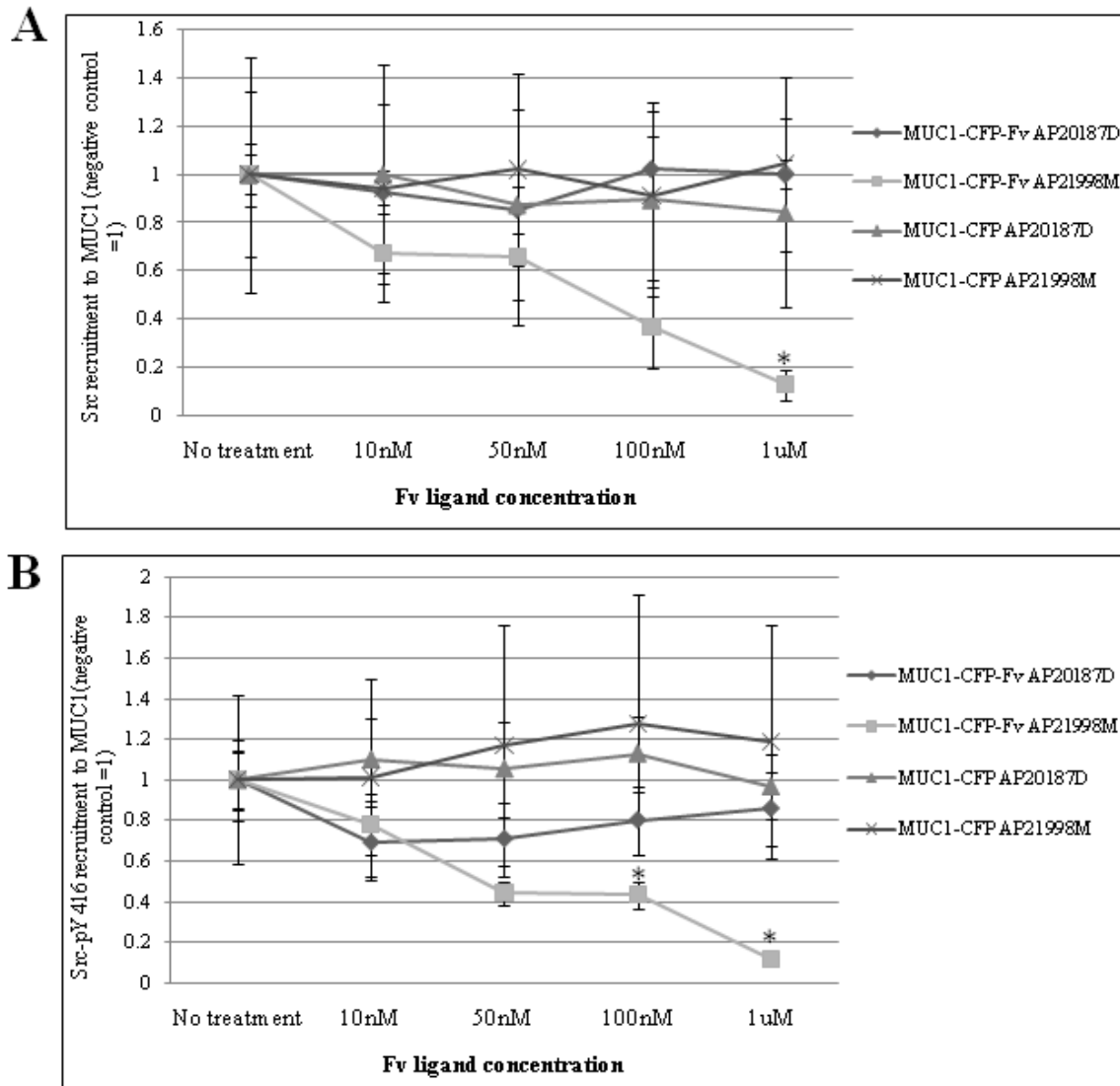


Figure 4.6. Densitometric analysis of Src and Src^{P416} recruitment to MUC1-CFP-Fv and MUC1-CFP after treatment with Fv domain ligands demonstrates significant reduction in Src and Src^{P416} recruitment to MUC1-CFP-Fv after AP21998^M treatment. Densitometry was performed on Src (A) and Src^{P-Y416} (B) with normalization to MUC1-CD as a loading control. No treatment conditions were set to one for each data set and the remaining conditions expressed as a ratio. Asterisk indicates conditions which do not overlap with any others in the data set ($p < 0.05$).

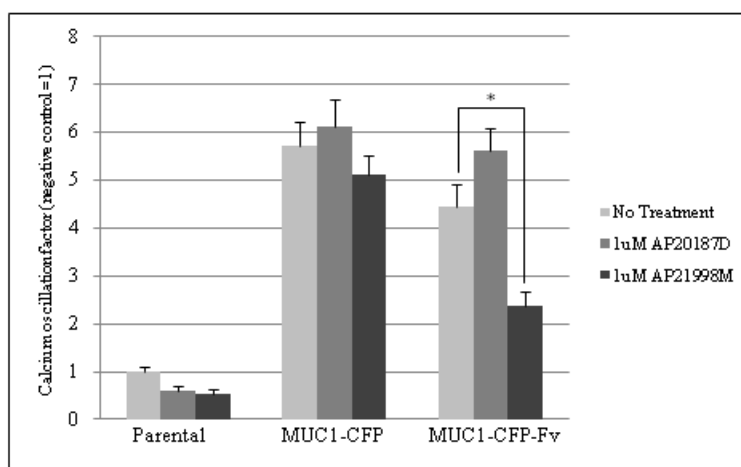


Figure 4.7. Disruption of MUC1-CD dimerization results in inhibition of ICAM-1 binding induced calcium oscillations. HEK 293T (parental), MUC1-CFP, and MUC1-CFP-Fv cells were analyzed for ICAM-1 binding induced calcium oscillations following pre-treatment with 1μM AP20187^D, 1μM AP21998^M, or no treatment control. Parental No treatment condition was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates pairs in the data set which are statistically different ($p < 0.05$).

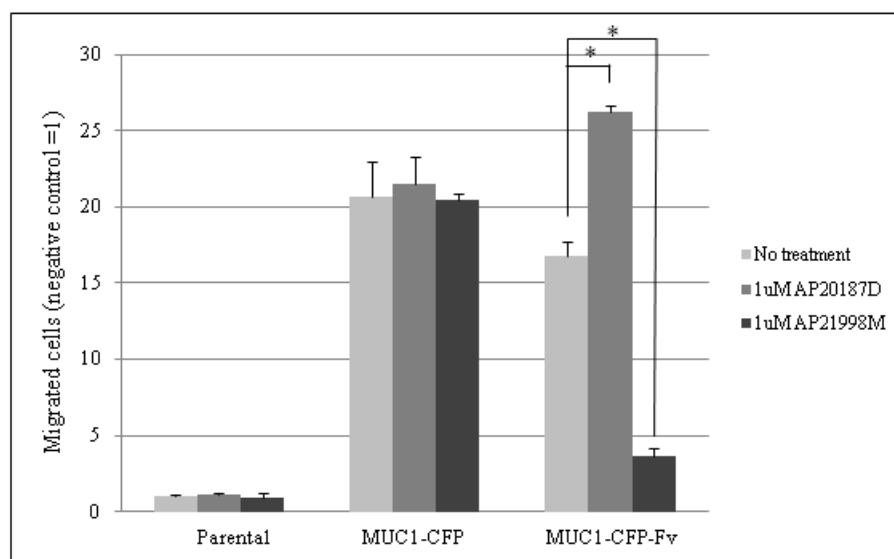


Figure 4.8. Disruption of MUC1-CD dimerization results in inhibition of ICAM-1 binding induced cell migration. HEK 293T (parental), MUC1-CFP, and MUC1-CFP-Fv cells were analyzed for migration through an NIH ICAM-1 positive monolayer following treatment with 1 μ M AP20187^D, 1 μ M AP21998^M or no treatment control. Parental No treatment condition was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates pairs in the data set which are statistically different ($p < 0.05$).

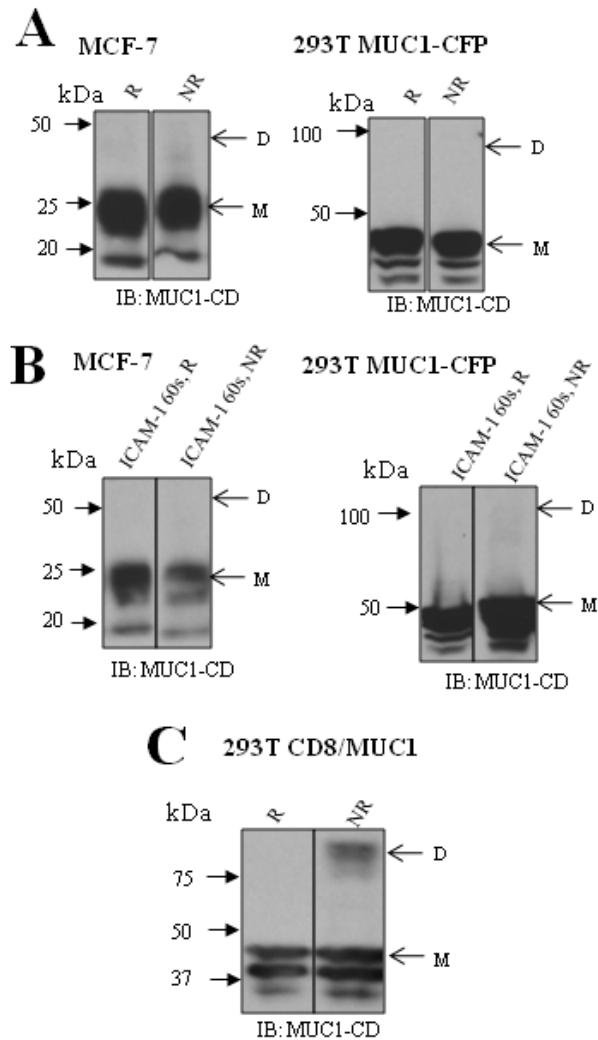


Figure 4.9. MUC1 cytoplasmic domain does not form covalently linked dimers constitutively or following ICAM-1 ligation. Human breast cancer cell line MCF-7 (left panels) and 293T MUC1-CFP transfectants (right panels) were assayed for constitutive (A) and 60 second ICAM-1 binding induced (B) covalently linked dimers. Reducing (R) and non-reducing (NR) conditions were run on separate SDS-PAGE gels followed by probing with anti-MUC1-CD. 293T CD8/MUC1 transfectants were included as a positive control (C). “D” and “M” indicate expected molecular weights of dimer and monomer, respectively.

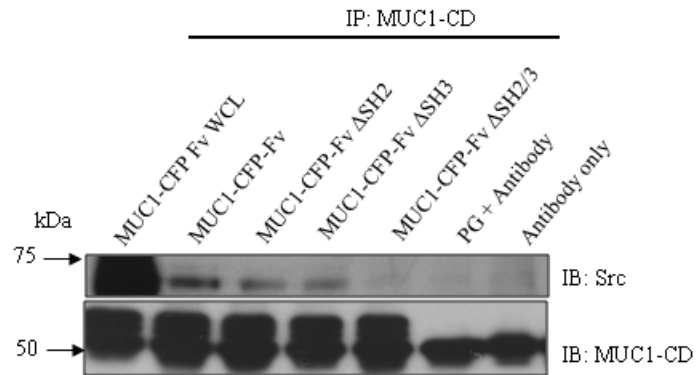


Figure 4.10. MUC1-CD contains SH2 and SH3 binding domains for Src kinase. MUC1-CFP-Fv cells with mutations of the SH2 (Y⁴⁶F; ΔSH2) and/or the putative Src SH3 binding sites (P³⁷A/P³⁸A; ΔSH3) were assayed for Src recruitment to MUC1-CD. Immunoprecipitation with anti-MUC1-CD was followed by SDS-PAGE and probing with anti-Src, and anti-MUC1-CD (as a loading control) with stripping of blots between each probe. Whole cell lysates (WCL), PG (Protein G) + Antibody and Antibody only lanes are included as controls.

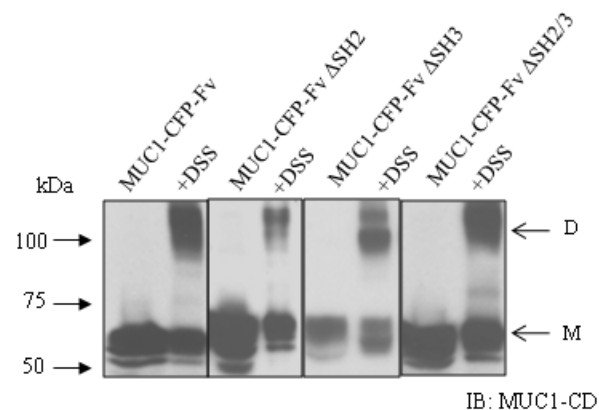


Figure 4.11. MUC1-CFP-Fv SH2 and SH3 binding domain mutants form MUC1-CD dimers. MUC1-CFP-Fv cells with mutations of the SH2 (Y⁴⁶F; ΔSH2) and/or the putative Src SH3 binding sites (P³⁷A/P³⁸A; ΔSH3) were assayed for MUC1-CD dimerization by treatment with DSS. Following treatment, cells were run on SDS-PAGE and probed with anti-MUC1-CD. “D” and “M” indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates are included as controls.

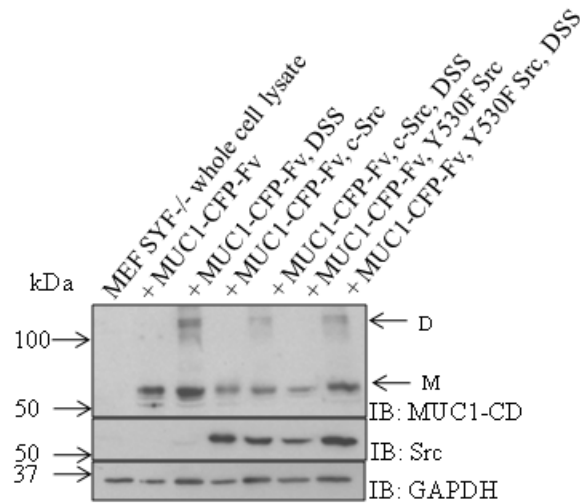


Figure 4.12. MUC1-CFP-Fv forms cytoplasmic domain dimers in MEF SYF^{-/-} cells. MEF SYF^{-/-} cells, lacking Src family kinases, were transfected with MUC1-CFP-Fv alone or in combination with Src or Src Y530F, treated with DSS, and assayed for MUC1-CD dimerization. GAPDH is included as a loading control. “D” and “M” indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysate (WCL) lanes are included as controls.